if any N is split off in the conversion, it is less than 0·5 % of the original N. The value reported represents a solubility of 0·0170 mg. N per ml. or 0·102 g. protein per litre. Hsu & Wu's values when converted to the same basis show close agreement, 0·017 ± 0·006 mg. per ml.[1]

It can be observed that when the progress curve is drawn to allow for the solubility of the fibrin, the curve is that typical of many enzyme reactions. This is in contrast to the results of Klinke & Elias [1931] and Kugelmass [1925] who found the reaction to be autocatalytic. The difference may be due to the different physical conditions used.

Fibrinolytic action of purified thrombin. A number of reports have appeared in the literature suggesting that thrombin has a fibrinolytic action (see Hirose). The most conclusive is that of Hirose [1934]. She reported that using sterile thrombin and fibrinogen, fibrinolysis occurred in 3 to 6 days. To our surprise, we found that our purified thrombin had a very high fibrinolytic action under the conditions of our experiments, measurable fibrinolysis occurring 4 hr. after the addition of thrombin. Fig. 3 shows an experiment of this nature. The fibrin formed reached a maximum in 3 hr. and then rapidly decreased. Increasing the thrombin concentration decreased the total amount of fibrin formed and increased the rate of fibrinolysis. Controls showed that it was not due to the shaking, and that removal of the supernatant containing most of the thrombin at the end of clotting resulted in the fibrinolysis being decreased proportionately. That such a marked action of thrombin has not been observed before may be due to the use of less purified reagents. As found by Hirose, the product of the reaction appears to be Hammarsten's fibrinoglobulin, no non-protein nitrogen appearing during the reaction.

Fig. 3. Fibrinogen N = 1·09 mg. Thrombin N in *A*, 0·064 mg.; in *B*, 0·128 mg.

It is difficult to prove that the fibrinolytic enzyme is actually thrombin and not another enzyme which has accompanied it through the purification process. As Mellanby's preparation of thrombin involves the preparation of prothrombin followed by its spontaneous activation and the removal of impurities which do not undergo the same change in properties, it seems unlikely that the fibrinolysis is due to an accompanying enzyme.

This work was originally undertaken with a view to applying the method (determination of the fibrin nitrogen by micro-Kjeldahl analysis) as a quantitative basis for the study of various problems in blood clotting. This has not proved feasible chiefly because of the difficulty in stopping the reaction to take samples. Any other method than centrifuging results either in the precipitation of the fibrinogen or the re-solution of the fibrin.

[1] A further indirect proof of the solubility hypothesis is the solubility of fibrinogen in salts used as precipitants. Campbell & Hanna [1937] have recently shown that fibrinogen is precipitated with 12·5% sodium sulphite so as to yield values which check with the Van Slyke & Cullen method of estimating fibrin. In a series of experiments the solubility of fibrinogen under the conditions of their method was found to be 0·043 mg. N per ml., the equivalent of 0·014 mg. fibrin N per ml. in the Van Slyke & Cullen method.
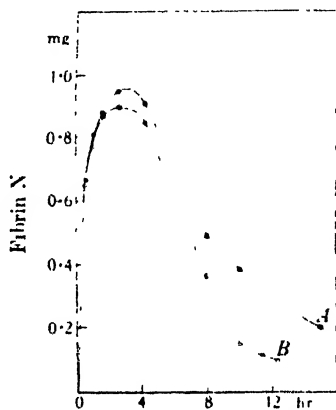
SUMMARY

When thrombin acts on a solution of fibrinogen, free from other proteins, all the fibrinogen N appears as fibrin nitrogen. Owing to the solubility of fibrin, however, as much as 10 % of the fibrin nitrogen may be present in the supernatant. Purified thrombin preparations have a marked fibrinolytic action.

Note added 5 July, 1938. Since this paper was sent to press the results of a similar study by Presnell (*Amer. J. Physiol.* **122**, 596, 1938) have been published. When his results are analysed, they indicate an interpretation similar to that given above, namely that the difference in the fibrinogen nitrogen is due to the solubility of fibrin rather than to a "proteolytic" action of thrombin.

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CLVIII. ENZYME SYSTEM OF
BACT. SUBOXYDANS

II. EFFECT OF ACIDS AND pH

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It has been shown [Butlin, 1936, 1938] that the addition of chalk to maize wort agar used as growth medium for *Bact. suboxydans* has a striking effect on that organism's enzymic activity. This effect was ascribed to the neutralization by the chalk of the acid formed by the growing culture on the medium. To obtain further evidence for this, the direct action of acids on the highly oxidizing suspension derived from chalk-containing maize wort agar was examined.

EXPERIMENTAL

A suspension of *Bact. suboxydans* (dry weight 25 mg. ml.) was prepared as previously described [Butlin, 1936], after 2 days' growth at 30 on maize wort agar containing 2 % chalk. 4 ml. portions of the main suspension were treated with 1 ml. acid of various strengths, left for $\frac{1}{2}$ hr., centrifuged and washed twice with sterile saline, and finally made up with 6 ml. sterile saline. The control received the same treatment except that water was substituted for acid. The O_2 uptake and CO_2 production with 0·3 ml. 0·1 M glucose were then determined in the Barcroft differential manometer by the usual methods, phosphate buffers of pH 6·0 and a temperature of 30 being used throughout.

Figs. 1 and 2 contain the O_2 uptake-time and CO_2 evolution-time curves with glucose as substrate given by suspensions treated with varying concentrations of HCl. The latter are expressed in terms of the actual strength of acid in contact with the bacterial cells during treatment. It should be emphasized that after treatment the cells were well washed and their action with glucose conducted at pH 6·0.

The results obtained bear a strong resemblance to those showing the effect of time of growth of the culture on the medium [Butlin, 1938], except that there was no initial increase in activity. The control suspension gave the normal behaviour of *Bact. suboxydans* with glucose at pH 6·0, i.e. rapid initial O_2 uptake, relatively slow initial evolution of CO_2, followed by an increase in the latter until its rate of evolution became equal to the rate of O_2 uptake. Treatment of the suspension with 0·001 N HCl had only slight effect, but with increasing concentration of acid the O_2 uptake and evolution of CO_2 decreased by approximately equal amounts until the latter entirely ceased, when the O_2 uptake (470 μl.) was equivalent to slightly less than 1$\frac{1}{2}$ atoms O for each molecule of glucose. This condition, in which the reaction with glucose was reduced to a simple oxidation process, was attained by treatment of the suspension with 0·006 N HCl. From this stage the effect of treatment with more acid was less intense and a concentration as great as 0·02 N was required to reduce the O_2 uptake to the equivalent of

1 atom O (344μl.). Despite the large decrease in total O_2 uptake and the total inhibition of CO_2 production, it is clear from Fig. 1 that the initial rate of O_2 absorption, up to about $350\,\mu$l., is practically unaltered by treatment of the suspension with concentrations of HCl not greater than $0.02N$. From this it is
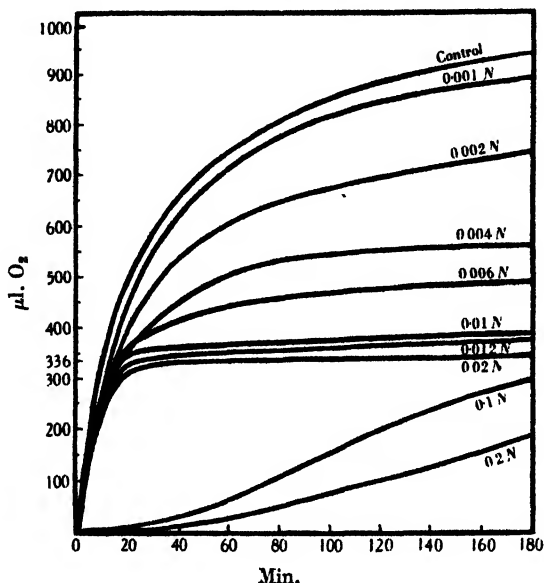

Fig. 1. O_2 uptakes, with 0.3 ml. $0.1\,M$ glucose as substrate, of suspensions of *Bact. suboxydans* treated with different concentrations of HCl and then washed. Temp. $30°$. pH 6.0.

Fig. 2. Evolution of CO_2, with 0.3 ml. $0.1\,M$ glucose as substrate, of suspensions of *Bact. suboxydans* treated with different concentrations of HCl and then washed. Temp. $30°$. pH 6.0.
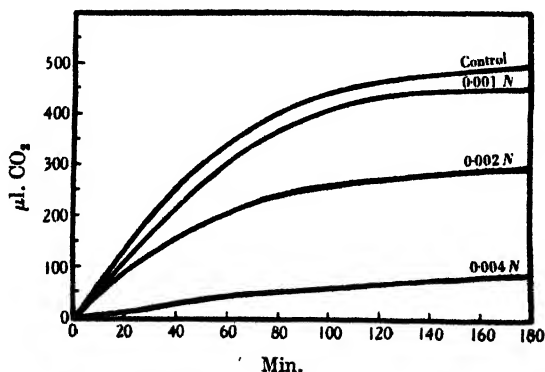
inferred that the rapid initial O_2 uptake is chiefly the result of a simple oxidation process. With concentrations of HCl greater than $0.02N$ the O_2 uptake was gradually inhibited until there was complete aerobic inactivation after treatment with $0.4N$ acid. The variation with different concentrations of acid of total O_2 uptake and CO_2 production given by the treated suspensions with glucose after 3 hr. in the manometer is shown in Fig. 3. The rapid fall in the curve at the beginning illustrates the relatively great effect of the smaller concentrations of

acid, which decrease O_2 uptake and CO_2 evolution by equal amounts, up to the point when the CO_2 production is completely inhibited ($0.006N$ HCl). After this stage there is much greater resistance to the effect of increasing acid concentration, shown by the much flatter portion of the curve at an O_2 uptake equivalent approximately to 1 atom O. The real effect is even greater than is apparent from Fig. 3 owing to the necessity for telescoping the scale for the larger acid concentrations.
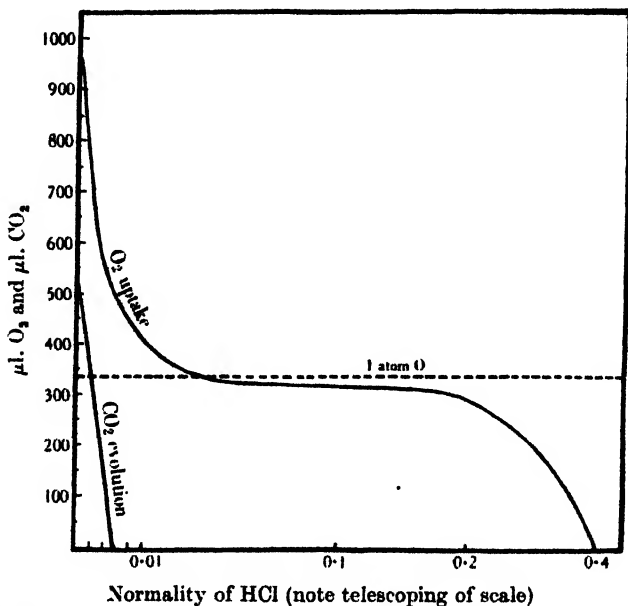

Normality of HCl (note telescoping of scale)

Fig. 3. O_2 uptake and CO_2 evolution, after 3 hr. in manometer with 0·3 ml. 0·1 M glucose as substrate, of suspensions of *Bact. suboxydans* treated with different concentrations of HCl and then washed. Temp. 30°. pH 6·`.

These results indicate strongly a differential action of acid on the enzymic system of *Bact. suboxydans* and constitute further evidence for the conclusions reached in a previous paper [Butlin, 1938]. There it was suggested that *Bact. suboxydans* possesses an enzyme system containing at least two important components. One, the more sensitive to acid, catalyses a reaction in which the O_2 absorbed is equal to the CO_2 evolved. The rapidly falling portion of the curve in Fig. 3, where the O_2 uptake and CO_2 evolution decrease by equal amounts when the suspension has been treated with comparatively small concentrations of acid, is contributory evidence for the existence of this component. The other component catalyses a simple oxidation of glucose to gluconic acid in which 1 atom O for each molecule of glucose is absorbed and a partial further simple oxidation to 5-ketogluconic acid. This is represented by the flatter portion of the curve, which clearly illustrates its resistance to the action of acid.

These data were obtained with HCl. Gluconic acid behaved in a similar manner.

Influence of pH

The above results may be regarded not merely as the result of the action of acid on the enzyme system of *Bact. suboxydans* but also as the consequence of subjecting the suspensions to various pH levels for a certain period, the cells

being then washed and their action on glucose determined at pH 6·0. Fig. 4 gives the pH produced by the addition of increasing concentrations of HCl to the suspensions, which consisted of cells in physiological saline, and to saline alone. The buffering action of the cells is apparent from the figure.
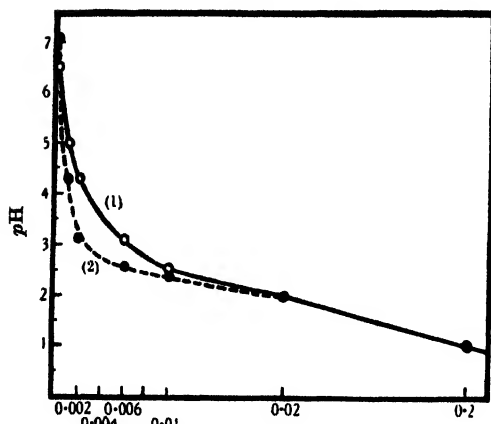
Fig. 4.

Fig. 5.

Fig. 4. pH produced by different concentrations of HCl. (1) o—o On suspensions of *Bact. suboxydans* in saline. (2) •·····• on saline alone.
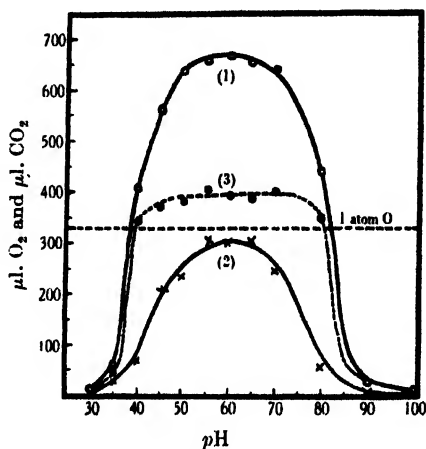
Fig. 5. (1) O_2 uptake o—o; (2) CO_2 evolution ×—× and (3) "O_2 uptake unaccompanied by CO_2 evolution" after 1 hr. in manometer with 0·3 ml. 0·1 M glucose as substrate, given by suspensions of *Bact. suboxydans* at different pH's.

The results obtained by the action of acids gave evidence for the presence of two components in the enzyme system of *Bact. suboxydans*. Similar conclusions may be drawn from the values for O_2 uptake, CO_2 output and "O_2 uptake unaccompanied by CO_2 output" given with glucose by well washed suspensions of the organism at different pH after 1 hr. in the Barcroft manometer (Fig. 5). For pH 3·0, 4·0, 5·0 phthalate, for 6·0, 7·0 and 8·0 phosphate and for 9·0 and 10·0 borate buffers were used. A temperature of 30° was used throughout. The results show that, though there is little significant difference in CO_2 production or total O_2 uptake at pH 5·0, 6·0 and 7·0, there is a rapid decrease below 5·0 and over 7·0. On the other hand the values for "O_2 uptake unaccompanied by CO_2 output" remain practically constant between pH 4·0 and 8·0 at a value just above the equivalent of 1 atom O, whereas at pH 4·0 and 8·0 the CO_2 production is practically nil. Here again we have the phenomenon of CO_2 production ceasing when the reaction represented by "O_2 uptake unaccompanied by CO_2 output" is intact. This constitutes further evidence for the suggested two-component enzymic structure of *Bact. suboxydans* outlined above.

Cultures grown on other media

The evolution of CO_2 with glucose is not confined to suspensions derived from cultures grown on maize wort agar with or without chalk. In suitable conditions it has been observed with cultures from every other growth medium so far employed, e.g. glycerol-yeast agar with and without chalk, malt wort agar with and without chalk, bouillon agar with chalk, glycerol-yeast extract solution, 10 %

glucose-chalk-yeast extract solution and 10 % sorbose-yeast extract solution. This property of *Bact. suboxydans* is therefore not specific to cultures grown on maize wort agar but is a normal characteristic of the organism.

DISCUSSION

Previous experimental results [Butlin, 1938] suggested that *Bact. suboxydans* possesses an enzyme system containing not only a component catalysing a simple oxidation process without production of CO_2 but another component which catalyses a reaction in which the O_2 absorbed is equal to the CO_2 evolved. The work described in the present paper presents confirmatory evidence for this suggestion, based on the differential effect of pH and of the direct action of acid on the enzyme system.

In ordinary liquid culture *Bact. suboxydans* is characterized by its slight intensity of oxidation as exemplified in such typical and almost quantitative transformations as glucose to gluconic acid, sorbitol to sorbose and glycerol to dihydroxyacetone. It is therefore surprising that it should possess a highly oxidizing factor in its enzymic equipment as shown by the action of washed suspensions of the organism with glucose under Barcroft conditions. It might be suggested that the strain used in the experiments had altered in character since its isolation. Similar results, however, have been obtained from 5 cultures from different sources. Moreover, these highly oxidizing suspensions when inoculated into liquid cultures show the modified oxidations characteristic of the organism. A more probable explanation is that *Bact. suboxydans* is unable appreciably to exert its more highly oxidizing function because of the conditions it creates in the liquid cultures usually employed. This function, which is sensitive to acid conditions, will be inhibited if acid is formed in sufficient quantity, leaving intact the more moderately oxidizing component. This explanation is readily applicable to the case where glucose is the substrate in suitable liquid medium of initial pH 6·0: the rapid formation of gluconic acid by the moderately oxidizing component would quickly inhibit the more drastic oxidation. At first sight it does not appear to explain the almost quantitative production of sorbose from sorbitol and dihydroxyacetone from glycerol, the chief final products being neutral, when suitable concentrations of the substrates in yeast water at pH 6·0 are inoculated with suspensions of *Bact. suboxydans*. In practice, however, it is found that owing to the formation of some unknown acid the pH of these solutions falls rapidly to 4·5 and often lower, and at these levels only the less actively oxidizing component is able to function. *Bact. suboxydans*, therefore, while possessing a highly oxidizing component in its enzyme system, is usually unable appreciably to utilize it and is chiefly noted for its property of restricted oxidation.

It is possible that the CO_2 production with its accompanying equal O_2 absorption is due to some anabolic process of the nature suggested by Barker [1936], Giesberger [1936] and Clifton [1937], in which the substrate is converted into cell materials simultaneously with the normal oxidative process. This possibility is being investigated.

SUMMARY

From further evidence based on the differential effect of pH and of the direct action of acid on the aerobic activity of washed suspensions, it is concluded that *Bact. suboxydans* possesses an enzyme system containing at least two important components. One, less sensitive to acid but more rapid in its action, catalyses a simple oxidation process. The other component catalyses a reaction in which the O_2 absorbed is equal to the CO_2 evolved.

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CLIX. CHOLESTEROL METABOLISM

I. ACIDS APPARENTLY CONCERNED IN THE METABOLISM OF CHOLESTEROL

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THE question of the origin of cholesterol in animals has been the subject of numerous researches but the problem of the metabolism of cholesterol has not commanded the same attention. Page & Menschick [1932] concluded that approximately 70 % of the cholesterol ingested by rabbits could not be accounted for by the sterol excreted in the faeces or by that stored in the animals. Schonheimer & Breusch [1933] found the cholesterol balance in mice to be negative or positive, depending on the supply of exogenous sterol. Beumer & Fasold [1933] showed that the rat also is apparently capable of metabolizing cholesterol. This conclusion was confirmed by Cook [1936, 1937], who found that rats fed a diet containing 2 % cholesterol were able to metabolize 20-30 % of the amount ingested.

This communication gives the results of further balance experiments. It is to be remarked that this work must be regarded as preliminary in nature and only indicative as to the possible fate of the cholesterol. Experiments on the acidic constituents of the faeces have yielded such consistent results that it is felt that, even if the direct relation of these acidic substances to cholesterol is not yet established, there is little doubt that certain fatty acids are concerned in, or associated with, cholesterol metabolism.

EXPERIMENTAL

The animals used were drawn from the laboratory piebald stock. As far as possible litter-mates were taken but if these were not available animals of approximately the same weights were used. The room in which the animals were kept was maintained at a constant temperature. The animals were kept in Hopkins metabolism cages, the urine and faeces being collected separately. The experiments can be divided into four dietary groups, three of which received different fats, and one no fat. The diets fed to the animals were made up as shown in Table I. In the diets of the control animals the cholesterol was omitted.

The cholesterol used was the specially recrystallized product of Glaxo, "Cholesterol G. L. Puriss. Recryst." The cholesterol was first dissolved in the fat portion of the diet by heating on a water bath, the molten mass being then well mixed with the other constituents.

The data with respect to the duration of the experiments, the weights of the animals and the amounts of food and cholesterol consumed are given in Table II. Exps. 1–4 belong to group A diet, exps. 5–7 to group B, Exps. 8 and 9 to group C and Exps. 10 and 11 to group D. Exps. 8 and 9 were done in collaboration with G. N. Jenkins. The results in Exps. 8 and 9, and Exps. 10 and 11 are the means of 3 animals in each case. In two instances, Exps. 1a and 6a, the animals died during the course of the experiment, and to counteract this effect as far as possible the

Table I. *Rats' diets*

All values in g.

Group and type of fat

	A Arachis oil	B Triolein (B.D.H.)	C Beef dripping	D Fat-free
Caseinogen	23·0	23·0	23·0	23·0
Rice starch	40·0	40·0	40·0	50·0
Cane sugar	17·0	12·0	17·0	22·0
Fat	15·0	20·0	15·0	0·0
Salt mixture	5·0	5·0	5·0	5·0
Yeast	7·5	7·5	7·5	7·5
Cod liver oil	2·5	2·5	2·5	0·0
Radiostoleum	0·0	0·0	0·0	1 drop per diem
Cholesterol (Glaxo)	2·0	2·0	2·0	2·0
Calories per 100 g. food	426·0	448·0	426·0	347·0

Notes. The caseinogen used was "Glaxo ashless casein E"; the salt mixture No. 185 of McCollum & Davis [1915]. The yeast was a dried yeast preparation of trade name "Ceremealo". No calorigenic value is given to the cholesterol.

Table II. *The duration of experiment, animals' weights, food and cholesterol consumptions of the experimental rats. In Exps. 8, 9, 10 and 11 three animals were used in each experiment. The weight figures are mean values.*

Exp. No.	Diet	Days on diet	Sex	Start	End	Gain	Total amount of food g.	Total amount of cholesterol fed g.
1 a	15% arachis oil	40*	♂	32	65	33	1618	—
b	controls	123		30	222	192		
2 a	Plus cholesterol	98	♂	32	176	144	1574	28·1
b		100		34	192	158		
3 a	15% arachis oil	123	♀	32	160	128	2015	—
b	controls			29	137	108		
4 a	Plus cholesterol	109	♀	29	116	87	1611	28·7
b				30	121	91		
5 a	20% triolein	29	♂	82	145	63	550	—
b	control			93	144	51		
6 a	Plus cholesterol	22*	♂	86	76	− 10	560	10·0
b		47		95	157	62		
7 a	Plus cholesterol	34	♂	44	97	53	503	9·0
b				39	92	53		
8	15% beef dripping controls	22	♀	193	187	− 6	715	—
9	Plus cholesterol	22	♀	212	231	19	818	14·6
10	Fat free diet	21	♀	104	117	13	645	—
11	Plus cholesterol	21	♀	97	133	36	657	12·0

Header for the "Animals" / "Wt. g." columns: Animals — Wt. g. — Start, End, Gain.

* Animal died.

remaining animals were allowed to continue for a longer time so that the food intake was approximately the same as for the other members of the series. The results for growth and food intake are in good agreement with the previously published figures.

The animals were bled under anaesthesia, the blood being analysed with the carcasses. The livers were removed from the well-bled animal and weighed. The values given for liver as % body weight are the weights of the blood-free livers as % of the gross weights of the animals just before death. The animals were gutted, the gut and contents being analysed together. This material was kept free from mesenteric fat as far as possible. The remaining portions were analysed as carcass. In some cases the livers were analysed individually.

Methods of analysis. A revision has been made in the methods of analysis. It was found that the Leathes & Raper method tended to give low and inconsistent results. The method adopted is modelled on that of Kumagawa & Suto [1908]. A prolonged alkaline hydrolysis is necessary, and the acidified mass must be extracted at least thrice with ether. The crude lipoid was then further saponified and the unsaponifiable matter extracted. The alkaline portion was then acidified and extracted with light petroleum, the acids being weighed. The lipoid in the case of the livers is the sum of the values obtained for the unsaponifiable matter (U.M.) and the fatty acids; on the other hand the values given for "fat" with the carcasses and gut represent the values obtained for the crude ether extract.

The cholesterol is estimated as the unsaponifiable fraction of the lipoids. The criticism might be made that this fraction is too ill-defined and includes compounds other than sterols. Preliminary experiments show, however, that the digitonin-precipitable material represents a high proportion of the U.M. (90 % for the cholesterol-fed animals). It should be pointed out that the digitonin method shows a specificity for the normal series of sterols only; the *epi* series is not estimated by this method. Also as will be seen later coprosterol would be estimated by this method. The point at issue has been to decide whether cholesterol has been converted into a substance which would not be estimated in the unsaponifiable fraction and on this point the experiments yield suggestive results.

The question of the formation of coprosterol from cholesterol has not been studied but it will be realized that such a transformation would not affect the total sterols in the unsaponifiable matter isolated. Coprosterol can be readily isolated from the U.M. of the control faeces by recrystallization from acetone: water is added to the material dissolved in acetone until the solution becomes cloudy; it is then warmed and allowed to cool slowly; the first slight flocculent precipitate is filtered off and the solution left to stand at 4°; after some hours the coprosterol crystallizes out in needles; the crystals are filtered off rapidly on a chilled filter. After one recrystallization from acetone a material was obtained giving the following analytical figures (Weiler): C, 83·09; H, 12·40 %. $C_{27}H_{48}O$ requires C, 83·33; H, 12·48 %. The M.P. was 93·5°, and although this is lower than the value for synthetic material (102°) the M.P. was not depressed on admixture with an authentic sample of synthetic coprosterol obtained from Dr Rosenheim.

It has been found impossible to use the colorimetric method for cholesterol with any degree of accuracy. Samples of the various fractions have been kept and their analysis will be the subject of a further communication.

Analyses of the animals

The results obtained for the analyses of the livers, carcasses and guts of the animals on the various diets are shown in Table III. It will be observed that when fat is included in the cholesterol-containing diet "fatty" livers result. On a fat-free diet there is no increase of fat in the liver. It will also be seen that the results obtained with the triolein-containing diet are not consistent. In Exp. 6a the animal died and the amount of fat in the liver had not increased. This animal

Table III. *The analyses of the animals for "fat" or total lipoid and unsaponifiable matter (U.M.) at the conclusion of the experiments*

Exp. no.	Diet	Sex	Wt. of animal g.	Liver Wt. g.	Liver As % body wt.	Liver "Fat" g.	Liver U.M. g.	Liver % "Fat"	Liver % U.M.	Carcasses Wt. g.	Carcasses "Fat" (a) g.	Carcasses U.M. (b) g.	a as % body wt.	b as % body wt.	Gut Wt. g.	Gut "Fat" g.	Gut U.M. g.
1	Control arachis oil	♂	222	7.73	3.50	0.293	0.026	3.79	0.34	162.0	30.53	0.149	18.85	0.09	17.25	4.97	0.070
2 a	Plus cholesterol	♂	176	13.69	—	1.553	0.451	11.35	3.29	302.0	59.70	0.184	19.70	0.06	21.0	3.76	0.086
b			192														
3 a	Control arachis oil	♀	160	5.81	3.60	0.165	0.010	2.84	0.17	272.0	47.80	0.216	17.50	0.08	20.4	1.80	0.060
b			137	5.89	4.30	0.213	0.018	3.62	0.31								
4 a	Plus cholesterol	♀	112	5.40	4.80	1.253	0.440	11.30	3.97	188.0	36.60	0.250	19.45	0.13	16.4	1.70	0.140
b			117	5.70	4.90												
5 a	Control triolein	♂	144	7.40	5.10	0.297	0.027	4.02	0.36	251.0	32.20	0.710	12.80	0.28	19.7	0.62	0.126
b			145	6.90	4.80	0.292	0.032	4.23	0.46								
6 a	Plus cholesterol	♂	76	3.00	3.95	0.129	0.039	4.10	1.30	56.4	1.33	0.265	2.35	0.47	4.7	0.17	0.027
b			157	8.40	5.35	0.982	0.255	11.70	3.04	138.5	24.00	0.470	17.30	0.34	10.8	1.34	0.193
7 a	Plus cholesterol	♂	97	7.59	7.80	0.752	0.074	5.54	0.55	150.0	14.80	0.268	9.85	0.18	17.2	0.90	0.096
b			92	6.24	6.75												
8	Control beef dripping	♀	188 mean	6.39 mean	3.40 mean	1.094	0.097	5.63	0.50	—	—	—	—	—	—	—	—
9	Plus cholesterol	♀	231 mean	10.20 mean	4.40 mean	4.361	1.214	13.80	3.88	—	—	—	—	—	—	—	—
10	Control fat-free	♀	117 mean	4.63 mean	4.00 mean	0.536	0.054	3.86	0.39	303.0	17.60	0.940	5.80	0.31	23.1	2.92	0.127
11	Plus cholesterol	♀	133 mean	5.16 mean	3.90 mean	0.647	0.056	4.17	0.36	333.0	33.25	1.380	10.00	0.415	34.3	7.75	0.343

had not eaten for some days before its death. In Exp. 7 the amount of fat in the liver has increased slightly but the amount is not comparable with that observed in Exp. 6. Exp. 7 was actually carried out first but it was uncontrolled. The animals showed a severe diarrhoea which fact probably accounts for the relatively small absorption. In Exp. 6 and with the control animals there was an initial period of diarrhoea which lasted for about a week but thereafter the animals, with the exception of no. 6a, adapted themselves to the diet.

It will be seen from the table, and with the reservations concerning the triolein-fed animals, that cholesterol is absorbed on a fat-containing diet irrespective of the type of fat fed. Also from the results of Exps. 2 and 4 there is apparently no difference in behaviour of the two sexes.

Analysis of the faeces

The faeces were collected over the whole period and finally dried at 70° to approximately constant weight. They were then weighed and powdered. An aliquot was extracted in a Soxhlet apparatus with ether for at least 72 hr. It has been found that more complete if somewhat more highly coloured extracts are obtained by using this solvent in place of light petroleum. The results for the analysis of the faeces are given in Table IV. They are in agreement with the

Table IV. *Details of the analyses of the lipoid and unsaponifiable matter (U.M.) in the faeces of the rats fed on the various diets*

Exp. no.	Diet	Dry wt. faeces g.	Aliquot estimation			Total in faeces		Lipoid as % faeces	U.M. as % lipoid
			Amount g.	Lipoid g.	U.M. g.	Lipoid g.	U.M. g.		
1	♂ controls arachis oil	59·7	20·0	2·62	0·93	7·8	2·97	13·0	38·1
			20·0	—	1·06				
2	Plus cholesterol	97·8	20·0	7·59	4·89	37·20	23·80	38·0	64·0
3	♀ controls arachis oil	79·4	20·0	2·16	0·84	8·60	3·23	10·8	37·6
4	Plus cholesterol	94·2	40·0	17·05	10·39	40·10	24·50	43·5	61·0
5	Controls triolein	21·9	20·0	2·71	1·26	2·96	1·38	13·6	46·5
6	Plus cholesterol	34·6	20·0	7·72	5·31	13·35	9·20	38·6	68·7
7	Plus cholesterol	29·3	29·3	9·92	6·63	9·92	6·63	33·9	66·7
8	Controls beef dripping	37·0	20·0	3·40	0·545	6·28	1·01	17·0	16·0
9	Plus cholesterol	52·1	20·0	7·96	3·82	20·74	9·96	39·8	48·0
10	Controls fat-free diet	22·2	10·0	0·79	0·45	1·75	1·00	7·9	57·0
11	Plus cholesterol	36·0	10·0	3·71	3·41	13·36	12·30	37·1	92·0

previously published results. The lipoid is the ether-soluble material. This was saponified for at least 5 hr. with 10% alcoholic KOH. The material was then diluted with water and extracted with ether. The ether extracts were washed 5 times with water. No soaps have been found in tested portions of the U.M. The remaining alkaline material was acidified with conc. HCl and the acids extracted with light petroleum. These results will be dealt with later.

Analysis of the urine

Qualitative experiments had indicated that the amount of cholesterol excreted in the urine of cholesterol-fed rats was not greatly augmented but no quantitative experiments had been made. The urines of the animals in Exps. 1, 2, 3 and 4 (Table II) were collected over the period of experiment and the results shown in Table V were obtained.

The urinary lipoids were estimated by first heating the urine on a water bath for 1 hr. with HCl and extracting with ether. The ethereal extracts were saponified and the U.M. extracted. The U.M. gave a precipitate with digitonin solution.

Table V. *Analyses of rats' urine*

Exp. no.		Vol. ml.	U.M. in aliquot	U.M. calculated in total vol. mg.
1	Normal	774	—	—
2	Cholesterol	596	—	—
3	Normal	1706	270 ml. gave 8·8 mg.	55·5
4	Cholesterol	630	200 ml. gave 17·4 mg.	54·8

A point of interest is the decrease in the urine volume of the cholesterol-fed animals. It was also noticed that the urine was much darker in colour. The results indicate that excretion of U.M. in the urine is a negligible factor in the metabolism of cholesterol although the reservation must be made that other breakdown products may be present.

The cholesterol (as U.M.) balance sheet

From the values that have been obtained it is now possible to construct a balance sheet (Table VI).

Table VI. *The U.M. balance sheet*

The values for U.M. in the table have been obtained by subtracting from the U.M. of the cholesterol-fed animals the amount of U.M. present in the control animals.

U.M. in	Arachis oil		Triolein Exp. 6	Beef dripping	Fat-free
	♂ g.	♀ g.	g.	g.	g.
Faeces	20·83	21·27	7·82	8·95	11·30
Livers	0·43	0·41	0·24	1·12	0·00
Carcasses	0·04	0·03	0·03	—	0·44
Gut	0·02	0·08	0·09	—	0·22
Total	21·32	21·79	8·18	10·07	11·96
Cholesterol fed	28·10	28·70	10·00	14·60	12·00
% accounted for	76·00	76·00	82·00	69·00	99·70

These results show that when fat is included in the diets of cholesterol-fed rats there is a loss of cholesterol irrespective of the type of fat fed or the amount of cholesterol. The loss of cholesterol varies between 20 and 30 %. The higher value was obtained in Exp. 8 and here the results are not complete as the U.M. was not determined in the carcasses or gut. It is extremely probable that here a higher percentage of cholesterol would have been recovered had these values been determined. The results for the fat-free diet are of interest. It will be seen that here there is virtually complete recovery of the cholesterol fed. The value for the carcass U.M. is rather high and might possibly be correlated with the fact that the cholesterol-fed animals grew better than the control animals. The results make it exceedingly probable that in the rat cholesterol is only metabolized in the presence of fat. It would also appear that the type of fat fed has but slight effect on the metabolism of cholesterol.

The fate of the cholesterol

Some indications of a possible fate of the cholesterol or some association with acidic substances may now be given. Table VII is what might be called the lipoid balance sheet of cholesterol metabolism as reflected in the faeces. It would appear from the results that have been given that the lipoid constituents of the carcasses do not give any clue as to the fate of the cholesterol.

In column A are the values for the extra faecal lipoids due to or caused by cholesterol feeding. In column B are the values for U.M. These figures appear in

Table VII. *The faecal lipoid balance sheet showing the difference in the distribution of the faecal lipoids of the control and cholesterol-fed animals on the various diets*

All values in g.

Exp. nos. and diet	Lipoid			U.M.			Diff. between A and B C	Acids			Cholesterol			
	Controls	Plus cholesterol	Diff. A	Controls	Plus cholesterol	Diff. B		Controls	Plus cholesterol	Diff. D	Fed	Accounted for as U.M.	Metabolized E	% metabolized
1 and 2 Arachis oil ♂	7·80	37·20	29·40	2·97	23·80	20·83	8·60	4·45	10·10	5·65	26·10	21·32	6·78	24·1
3 and 4 Arachis oil ♀	8·60	40·10	31·50	3·23	24·50	21·27	10·20	5·23	10·38	5·15	28·70	21·79	6·91	24·1
5 and 6 Triolein	2·96	13·35	10·40	1·38	9·20	7·82	2·58	1·41	3·44	2·03	10·00	8·18	1·82	18·2
8 and 9 Beef dripping	6·28	20·74	14·46	1·01	9·96	8·95	5·51	4·80	8·25	3·45	14·60	10·10	4·50	30·4
10 and 11 fat-free	1·75	13·36	11·61	1·00	12·30	11·30	0·31	0·73	0·97	0·24	12·00	11·96	0·04	0·3

the U.M. balance sheet as faecal U.M. The difference between A and B shown in column C would therefore represent material other than U.M. caused to be excreted in the faeces by cholesterol feeding. This may be regarded as unaccounted lipoid. It will be obvious that this material should be present in the alkaline fraction as the potassium salt of an acid. If the alkaline fraction is acidified and extracted with light petroleum it is found that "fatty" acids are indeed present. In column D are given the differences between the amount of acids found in the faeces of the cholesterol-fed rats and those of the control animals. This can be regarded as the increase in the amount of acids caused by cholesterol feeding. In column E are given the values for the amount of cholesterol metabolized. This was obtained by subtracting the amounts of cholesterol recovered as shown in the balance sheet (Table VI) from the total amount of cholesterol fed in each separate series.

It will be seen from the table that the values shown in colums C, D and E in each experiment are of the same order. In Table VIII are given the results of previously published experiments [Cook, 1937] to which the amounts of acid have been added. These experiments are not complete because the acid was not estimated in the first series of faeces collected. A calculated value has therefore been added. The extraction of the faeces in these experiments was made with light petroleum.

Table VIII. *The lipoid balance in the faeces on varying fat concentrations plus cholesterol*

Diet fat %	Cholesterol			Lipoid unaccounted for g.	Acid found g.	% cholesterol metabolized
	Fed g.	Found in faeces and animals g.	Difference g.			
15	30·19	18·90	11·29	7·62	6·62	37·4
20	29·77	20·17	9·60	9·75	7·13	32·2
30	26·99	18·95	8·04	9·17	8·19	29·8
Totals			28·93	26·54	21·94	
Add calculated acid for first series					4·25	
					26·19	

It will be seen that here, as in the experiments described above, the missing cholesterol bears some relation to the production of acids which appear in the

faeces. It will be realized that if these acids are derived from cholesterol this might be reflected in an increase in the mean mol. wt. or equiv. wt. of the acids. The equiv. wt. has been determined by titrating with standard alkali in alcoholic solution at 60°. The results obtained are shown in Table IX.

Table IX. *The equiv. wt. of the mixed faecal fatty acids*

	Arachis oil		Triolein			Beef dripping		Fat-free	
Controls	320	316	354	359	350	285	303	323	332
	333	320							
	Mean 322		Mean 354			Mean 294		Mean 327	
Plus cholesterol	378	375	320	328	323	329	333		
						329	320	340	
	Mean 375		Mean 323			Mean 328			

It must be emphasized that the values given are those of the mixed fatty acids and the results are comparative only. It will be seen that with the exception of the triolein and fat-free diets there is a tendency for the equiv. wt. of the acids to rise on cholesterol feeding. On the triolein and fat-free diets the equiv. wts. of faecal acids from the control animals are high in value. It is possible that with the arachis oil and beef dripping diets there is a selective excretion of higher fatty acids in association with the cholesterol. The appearance of these fatty acids was first observed on the arachis oil diet. The mixed acids were crystallized out of alcohol and on recrystallization an acid of M.P. 73·5° was obtained. On analysis this compound gave (Weiler): C, 78·86, 78·69 and 78·53, 78·58; H, 13·01, 12·97 and 13·03, 12·97 %. The mol. wt. (Rast) was 404 and the equiv. wt. 386 and 390. The compound did not take up bromine in alcoholic solution. The formula $C_{26}H_{50}O_2$ corresponds with the analytical figures but the substance is probably a mixture of high fatty acids. It is possible that this acid is derived from the "lignoceric acid"[1] which is present in relatively large amount in arachis oil. No systematic fractionation of the faecal acids has yet been made. A preliminary Twitchell separation indicates that the saturated acids form a high percentage of the material.

The results with the other fats cannot be so easily explained. The concentration of lignoceric acid in beef dripping is very low and triolein naturally does not contain any. The position is far from clear but it would appear that the excretion of cholesterol in the faeces, and possibly its metabolism, are associated with the appearance of acids of high mol. wt.

SUMMARY

1. Rats have been fed on synthetic diets containing cholesterol in the presence of arachis oil, beef dripping and triolein. The cholesterol was also fed with a fat-free diet.

2. Cholesterol is absorbed irrespective of the type of fat fed. It is not absorbed on a fat-free diet.

3. Balance sheets of the cholesterol recovery on the various diets have been prepared. There is complete recovery on the fat-free diet but on the fat-containing diets only 70–80 % of the cholesterol can be recovered.

4. A lipoid balance sheet of the faecal excretion has been constructed. This indicates apparently that cholesterol metabolism is bound up with an increase of fatty acids in the faeces. The amount of cholesterol missing is approximately

[1] The position with regard to this acid in naturally occurring substances is well described by Chibnall *et al.* [1936].

equal in amount to the acids recovered. These acids are of high mol. wt. but it cannot be stated as yet that they are derived from cholesterol.

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CLX. COENZYME FACTOR OF YEAST

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ANIMAL tissues have been shown to contain coenzyme factor, an enzyme which catalyses the oxidation of coenzymes I and II by carriers [cf. Dewan & Green, 1937; 1938; Adler *et al.* 1937]. In this communication evidence is presented of the existence of the same enzyme in baker's yeast and other micro-organisms. The wide distribution of coenzyme factor offers additional evidence that it is the normal physiological agent for catalysing the oxidation of the pyridine nucleotides.

I. *Preparation of yeast coenzyme factor*

There are two methods available for extracting enzymes from yeast. The first and standard method is to dry yeast slowly, resuspend in water and allow autofermentation to proceed for some hours. By this procedure the more stable enzymes can be extracted. The method however is not applicable to the fragile enzymes like coenzyme factor and cytochrome oxidase which are destroyed in the course of drying the yeast. A more suitable method for obtaining these enzymes is that of the wet crushing mill (for a detailed description of the mill, cf. Booth & Green [1938]).

A suspension of baker's yeast (1 part yeast cake to 1 part water) was thoroughly ground in the roller mill and then centrifuged for 20 min. The supernatant liquid was mixed with $\frac{1}{2}$ vol. saturated $(NH_4)_2SO_4$ (*p*H 5) and was centrifuged. The supernatant fluid was discarded and the sediment was redissolved in the original volume of water. The precipitation with $(NH_4)_2SO_4$ was repeated twice. The precipitate was finally dissolved in the original volume of phosphate buffer, *p*H 7·2. By this procedure the coenzyme factor was obtained free from the soluble coenzyme dehydrogenases and from flavoprotein. Like its counterpart in animal tissues the yeast coenzyme factor is associated with highly peptized insoluble particles. As purification proceeds the insolubility of the particles becomes more noticeable. Sedimentation of the particles after the $(NH_4)_2SO_4$ procedure becomes feasible even in salt-free solution.

II. *The catalytic effect of yeast coenzyme factor on animal coenzyme dehydrogenase systems*

The coenzyme dehydrogenases catalyse the oxidation of their respective substrates by one of the two pyridine nucleotides. The reduced coenzyme is non-autoxidizable and its reaction with O_2 can only take place through the intermediation of some natural carrier such as cytochrome *a* or *b*, or an artificial carrier such as methylene blue. Coenzyme factor catalyses the reaction between reduced coenzyme I or II and the carrier. A system containing dehydrogenase, substrate, coenzyme, carrier and O_2 will not absorb O_2 unless coenzyme factor is added. Such a system is obviously suitable for testing the catalytic effect of coenzyme factor.

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The preparation of the lactic, malic and triosephosphoric dehydrogenases of animal source free from coenzyme factor has already been described by Dewan & Green [1938]. Table I shows the effect of yeast coenzyme factor on these three systems. We have also been able to demonstrate the factor effect in the hexose-monophosphoric dehydrogenase system of rabbit skeletal muscle. Our colleague H. S. Corran will deal with the action of coenzyme factor on this system in a separate communication.

Table I. *The catalytic effect of yeast coenzyme factor on the lactic, triosephosphoric and malic dehydrogenase systems of animal tissues*

μl. O_2 per 10 min.

	Lactic	Malic	Triose-phosphoric
Complete system	189	181	165
Without factor	9	10	0
Without coenzyme	0	0	0
Without substrate	5	0	21
Without dehydrogenase	3	6	0

The following quantities were used in the complete systems; *lactic*—1·5 ml. dehydrogenase, 0·2 ml. factor, 0·7 ml. 0·2% coenzyme I, 0·2 ml. 2M HCN, 0·05 ml. 0·5% methylene blue, 0·3 ml. $M/3$ pyrophosphate and 0·2 ml. M lactate; *malic*—same quantities except 0·2 ml. M malate instead of lactate; *triosephosphoric*—1·5 ml. dehydrogenase, 1 ml. factor, 0·7 ml. 0·2% coenzyme I, 0·2 ml. 0·5% methylene blue, 0·3 ml. $M/3$ pyrophosphate and 0·4 ml. $M/10$ hexosediphosphate (KOH in the centre pot).

III. *The catalytic effect of yeast coenzyme factor on yeast coenzyme dehydrogenase systems*

The malic, alcohol and triosephosphoric dehydrogenases were prepared from yeast by the following procedures.

Malic. The centrifuged juice of crushed baker's yeast was mixed at 0° with 3 vol. cold acetone. The precipitate was filtered and washed with acetone and ether. The dried powder was rubbed up with the original volume of water and the suspension was dialysed for 12 hr. at 0°. The precipitate was centrifuged and discarded. The solution was then treated for 10 min. at 52°.

Alcohol. Maceration juice of top brewer's yeast was diluted with 2 vol. water and dialysed for 6 hr. against running tap water. The centrifuged solution was then made 0·6 saturated with respect to $(NH_4)_2SO_4$, the precipitate collected by centrifuging and redissolved in the original volume of water. The $(NH_4)_2SO_4$ precipitation was repeated twice. The final solution was treated at 0° with an equal volume of cold acetone, and the precipitate was filtered and washed with acetone and ether. The dried powder was finally dissolved in the original volume of water.

Triosephosphoric. Dried baker's yeast was rubbed up with 10 vol. iced water and the suspension was centrifuged. This washing procedure was thrice repeated. The final suspension was made up by mixing 1 vol. of washed and centrifuged yeast cake with 2 vol. of water.

These three preparations contain highly active dehydrogenases free of coenzyme factor and flavoprotein. Table II shows the effect of addition of coenzyme factor to these dehydrogenase systems. It is clear that regardless of the source of the coenzyme dehydrogenase, whether from animal tissues or yeast, the reaction of reduced coenzyme I with methylene blue (or the natural carriers)

Table II. *The catalytic effect of yeast coenzyme factor on the malic, alcohol and triosephosphoric dehydrogenase systems of yeast*

| | $\mu l.\ O_2$ per 15 min. | | |
	Malic	Alcohol	Triose-phosphoric
Complete system	212	134	235
Without factor	29	17	26
Without coenzyme	0	0	10
Without substrate	0	0	22
Without dehydrogenase	0	0	0

The following quantities were used in the complete systems: *malic*—1·5 ml. dehydrogenase, 0·2 ml. factor, 0·7 ml. 0·2% coenzyme I, 0·2 ml. $2M$ HCN, 0·2 ml. 0·5% methylene blue, 0·3 ml. $M/3$ pyrophosphate and 0·2 ml. M malate; *alcohol*—1·5 ml. dehydrogenase, 0·5 ml. factor, 0·5 ml. 0·2% coenzyme I, 0·2 ml. 0·5% methylene blue, 0·3 ml. $M/3$ pyrophosphate, 0·2 ml. $M/2$ hydrazine and 0·1 ml. 98% alcohol; *triosephosphoric*—2 ml. yeast suspension, 0·5 ml. factor, 0·5 ml. 0·2% coenzyme I, 0·4 ml. $M/10$ hexosediphosphate and 0·3 ml. $M/3$ pyrophosphate (KOH in the centre pot).

takes place only in presence of the coenzyme factor. Furthermore, yeast factor works as efficiently with animal coenzyme dehydrogenase systems as does animal factor, and *vice versa*.

IV. *Comparison of yeast factor and flavoprotein*

Dewan & Green [1938] have shown that 1 mg. crude coenzyme factor of pig heart has the same activity as 20 mg. pure flavoprotein in the malic system (pig heart) and as 70 mg. pure flavoprotein in the triosephosphoric system (rabbit muscle). Tests with the crude yeast factor show that 1 mg. dry weight has the same activity as 6 mg. pure flavoprotein in the malic system (pig heart) and as 20 mg. pure flavoprotein in the triosephosphoric system (rabbit muscle). This discrepancy in no wise indicates that the coenzyme factors from the two sources are not the same. In the next section evidence is presented of the extraordinary similarity in properties of the enzymes from the two sources. It is important to note that the crude preparations of the factor whether from animal tissues or yeast contain all the insoluble enzymes and particles extracted from the cell. The coenzyme factor probably accounts for much less than 1% of the total dry weight. At this level of purity the absolute activity of the coenzyme factor is not very significant in a comparison of the enzymes prepared from different sources. What is significant however is that the catalytic activity of even the crude coenzyme factor is incomparably higher than that of pure flavoprotein.

The crude preparations of yeast factor were tested for the presence of flavoprotein with negative results. The fact that weight for weight the crude yeast coenzyme factor is many times more active than pure flavoprotein rules out all possibility that traces of flavoprotein could account for the catalytic effects. Flavoprotein was prepared from brewer's yeast by the method of Warburg & Christian [1932]. The flavin content was determined by extracting the dried powder with aqueous methyl alcohol and estimating the flavin content of the extract.

V. *Some properties of the yeast coenzyme factor*

Table III shows the extraordinary fragility of the yeast coenzyme factor. All these properties are identical with those of the coenzyme factor of pig heart. The yeast factor is also partially destroyed by drying and by precipitation with acetone.

Table III. *The effect of reagents and temperature on the activity of the yeast coenzyme factor*

Treatment	% loss in activity
10 min. at 52°	92
1 hr. at 38°	50
20 min. digestion with trypsin at 38°	100
3 min. at pH 4·0	75
3 min. at pH 4·6	0
3 min. at pH 9·0	90
12 hr. dialysis at 0°	10

Thus far we have been unable to detect any colour characteristic of the suspension of the coenzyme factor. The more concentrated suspensions are slightly brown but this colour does not change when the complete catalytic system is functioning. Dr Straub of the Molteno Institute kindly tested our preparations of the coenzyme factor for the presence of the coenzyme of the d-amino-acid oxidase. Only minute amounts were found to be present. As yet no direct evidence is available for assuming that the coenzyme factor contains the flavin-adenine coenzyme as its prosthetic group. Numerous attempts were made to resolve the coenzyme factor into a protein and crystalloidal component, but in no case was there evidence of such a resolution.

VI. *Coenzyme factor in other micro-organisms*

By a procedure similar to that employed in the case of baker's yeast and by the use of the roller mill it was possible to demonstrate the presence of coenzyme factor in the following micro-organisms: *Bact. coli*, *Bact. proteus* and *Bact. subtilis*. The most active preparation was obtained from *Bact. coli*.

SUMMARY

1. The preparation of coenzyme factor from crushed suspensions of baker's yeast and other micro-organisms is described.

2. Like its counterpart in animal tissues the enzyme catalyses the oxidation by carriers of the reduced diphosphopyridine nucleotide (coenzyme I).

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CLXI. THE CONVERSION OF COLCHICINE INTO COLCHICEINE

By ERIC BOYLAND AND ELINOR HUNTSMAN MAWSON

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COLCHICINE is readily converted into the corresponding hydroxymethylene derivative, colchiceine, by hydrolysis of the methoxymethylene group in dilute acid solution. Colchiceine has only a fraction ($\frac{1}{10}$ to $\frac{1}{200}$) of the biological action of colchicine [Brues & Cohen, 1936; Ludford, 1936; Boyland & Boyland, 1937]. Hence the activity of orally administered colchicine might be partially destroyed by conversion into colchiceine by the action of the acid in the gastric juice.

EXPERIMENTAL

Method of estimation

Colchiceine gives a green colour with $FeCl_3$ in chloroform; colchicine and hexahydrocolchicine [Bursian, 1938] give no colour under the same conditions. The test can be used as a qualitative test for the detection of colchiceine in samples of colchicine. The method described below has been developed for the colorimetric estimation of colchiceine. As the colour reaction is given with trimethylcolchicinic acid, this and other hydroxymethylene ketones would be estimated by this method.

The solution containing colchicine and colchiceine is extracted by shaking with three separate lots of 3 ml. chloroform. The combined extracts are placed in a 15 ml. graduated flask, and 4 ml. absolute alcohol and 0·8 ml. of a 0·2 % solution of $FeCl_3$ in chloroform added. The total volume is brought to 15 ml. by addition of chloroform. Standards for comparison are made by dissolving known amounts of pure colchiceine in chloroform. Such standard solutions are unstable and must be freshly made. The colours developed by the unknown and an appropriate standard are compared in a colorimeter, using a red screen if the amount is less than 1 mg. The depths of colour obtained are easily compared without a screen if the amount of colchiceine present is 1–4 mg.

The greatest error in the method lies in the extraction of colchiceine from the aqueous solution with chloroform. Extractions of pure colchiceine showed that between 90 and 95 % with an average of 93 % was extracted. Allowance is made for this error in the calculation of results.

Hydrolysis of colchicine

Colchicine $C_{22}H_{25}O_6N$, 1·5 H_2O should theoretically yield 92 % of its weight of colchiceine $C_{21}H_{23}O_6N$, 0·5 H_2O. Colchicine, on heating in $N/10$ HCl at 100° for 1 hr., gives only 93 % of the theoretical amount of colchiceine (see Fig. 1), even after allowing for the loss in the extraction. By carrying out the hydrolysis for 1 hr. and then estimating colchiceine the original colchicine content of a solution can be determined. Hydrolysis in stronger acid such as N HCl gives lower yields

(see Fig. 1) owing to further decomposition and a similar decomposition occurs even in weaker acid.

Solutions containing 1 mg. colchicine per ml. were incubated with N HCl, $N/50$ HCl and with normal human gastric juice at 37°. Samples of the incubated solutions were taken and colchiceine estimated by the method described. The hydrolysis curves obtained are shown in Fig. 2.
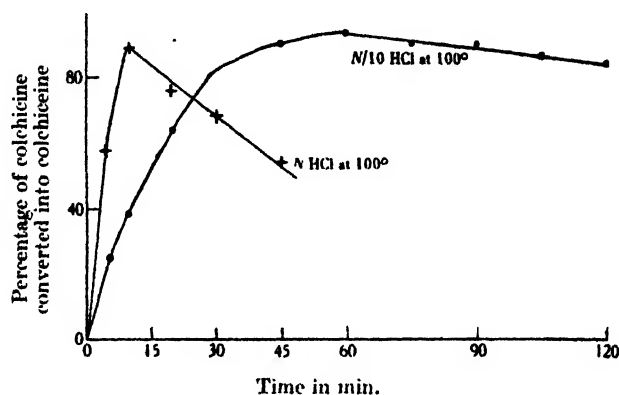
Fig. 1.
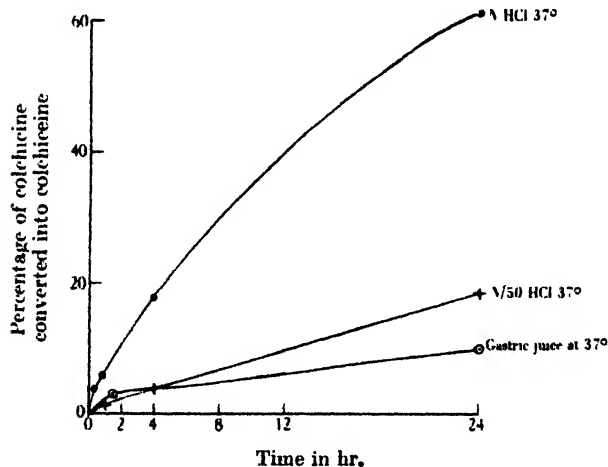
Fig. 2.

The amount of colchicine hydrolysed in $N/50$ HCl or by gastric juice during the time that orally administered colchicine would be expected to remain in the stomach is small. If colchicine is absorbed from the gut oral administration would be expected to be as effective as injection.

SUMMARY

A method for the colorimetric estimation of colchiceine and colchicine is described. The rates of acid hydrolysis of colchicine at 37° and 100° have been measured.

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CLXII. EXPERIMENTS ON THE CHEMOTHERAPY OF CANCER

I. THE EFFECT OF CERTAIN ANTIBACTERIAL SUBSTANCES AND RELATED COMPOUNDS

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MANY dyes containing aminonaphtholsulphonic acid residues have affinity for some cells contained in grafted tumours and lymph glands. Such dyes are isamine blue [Roosen, 1930], trypan blue [Opitz *et al.* 1926] and an azo dye formed from H-acid [cf. Kottman, 1935]. According to Ludford [1929] these dyes stain the macrophages of tumours rather than the malignant cells proper. Prontosil soluble, an antibacterial agent, contains an acetaminonaphtholsulphonic acid residue and on injection into animals with tumours it stains tumour tissue apparently in the same way as do the other dyes of this class. Prontosil soluble was found to have some slight inhibitory action on the growth of tumours in mice and many drugs related to this have been tested for their actions on the growth of tumours. The Schiff's base of 4:4'-diaminodiphenylsulphone and heptaldehyde was prepared and tested because heptaldehyde has been described as causing retrogression of spontaneous tumours [Strong, 1938].

The study of the effect of substances on tumour growth is much easier with grafted tumours as an unlimited number of comparable tumours can be obtained at any one time. With spontaneous tumours, however, each tumour must act as its own control and each has its own properties. It is also at present difficult to obtain sufficient spontaneous tumours.

Whereas grafted tumours become haemorrhagic on treatment with bacterial filtrates [Andervont, 1936] or colchicine [Boyland & Boyland, 1937] spontaneous tumours do not react in the same way. This difference in behaviour makes it appear possible that some substance might be effective against spontaneous cancers and not against grafted tumours. All substances mentioned in this paper have therefore been tested on both spontaneous tumours and grafted tumours but it is thought that more weight should be placed on the results with the spontaneous tumours.

EXPERIMENTAL

Benzidinesulphone was prepared as described by Culinane & Davies [1936]. Tolidinesulphone was prepared in the same way. Heptylideneaminobenzene-sulphonamide was prepared by the general method of Gray *et al.* [1937]. It was crystallized from alcohol and had M.P. 171–172°. (Analysis (Schoeller) found: C, 57·3; H, 7·55; N, 10·15%. $C_{13}H_{20}SO_2N_2$ requires C, 58·0; H, 7·5; N, 10·20%.)

Female mice with spontaneous tumours which were either detected among the old breeding mice of the Strong A or Dilute Brown strains or supplied by dealers were examined and the tumours measured with calipers three times per week. The length and breadth of the tumour was recorded and the sum plotted. Normally the growth of tumours plotted in this way is linear [Boyland & McClean, 1935]. After the rate of growth of a tumour had been recorded for such a time

that the sum of length and breadth had increased by about 10 mm. the animal was treated as shown in the figures with the compound under investigation. All the substances were administered orally using a syringe with a long blunt needle. The toxicity of each substance was determined and the daily dose given was less than 1/4 of the toxic dose. Each substance was ground with water and gum tragacanth to give a suspension 0·5 ml. of which contained the daily dose. The results of the treatment with each compound were observed with four mice with spontaneous tumours as shown in the tumour growth curves. On the death of each mouse the tumour or tumours were removed for histological examination and in all cases were shown to be mammary carcinomata. The results are shown in Figs. 1–23 and summarized in Table I. The figures marked (G) are taken from Gray et al. [1937]. If the size of the tumour was less at the end of the period of treatment than at the beginning the tumour is described as having retrogressed.

Each substance was also tested in a similar way on the growth of the Crocker Sarcoma 180 in mice of the Strong A or Dilute Brown strains. In this case five mice of the same strain were treated with the compound while five mice grafted with the same tumour at the same time served as controls. If the substance produced definite inhibition of growth this is indicated by + in Table I.

Table I. *Action of substances on tumour growth in mice*

Substance	Toxic dose mg.	Dose given mg.	Effect on spontaneous tumours. Each substance tested on 4 mice		Maximum survival under treatment days	Inhibitory action on grafted tumours (Crocker 180)
			No. of tumours showing			
			Inhibition of growth	Retro-gression		
Prontosil soluble	>50	25	3	2	16	−
Prontosil rubrum	2 (G)	3	3	0	50	−
p-Aminobenzenesulphonamide	100 (G)	25	4	2	58	+
p-Heptylideneaminobenzenesulphonamide	200	25	3	2	28	−
p-Aminobenzenesulphonyl-p'-sulphon-amidophenylamide hydrochloride	100 (G)	25	2	1	46	+
Sodium o-aminobenzenesulphonate	>200	50	2	2	21	+
Sodium m-aminobenzenesulphonate	>200	50	0	0	19	−
Sodium p-aminobenzenesulphonate	>200	50	4	1	43	−
Sodium sulphanilylsulphanilate	>200	20	4	3	33	+
Sodium p-hydrazinobenzenesulphonate	200	25	4	1	33	−
4:4'-Dinitrodiphenylsulphide	25	5	1	0	15	−
4:4'-Dinitrodiphenylsulphoxide	20	5	4	2	38	−
4:4'-Dinitrodiphenylsulphone	25	5	3	1	25	+
4:4'-Diaminodiphenylsulphide	30	5	3	1	35	.
4:4'-Diaminodiphenylsulphoxide	20	2	4	3	60	+
4:4'-Diaminodiphenylsulphone	10	2	4	2	16	+
4-p-Methoxybenzylidineamino-4'-amino-diphenylsulphone	200	4	3	3	15	+
4:4'-Diaminodiphenylsulphone glucose anil	100	10	4	2	37	+
Benzidinesulphone	20	4	3	0	44	+
Tolidinesulphone	10	4	3	2	15	−
Tetradecamethylene-1:14-diaminedihydro-chloride	2	1	3	2	25	+
Synthalin A (decamethylene-1:10-di-guanidine hydrochloride)	0·2	0·05	4	1	37	+
Synthalin B (dodecamethylene-1:12-di-guanidine hydrochloride)	0·2	0·05	2	1	32	−

Some of the compounds were tested for any action which they might have upon the increase in body weight of young mice. Groups of four mice weighing 10 g. each were weighed daily and while one group remained as control other

groups were given p-aminobenzenesulphonamide, 4:4'-diaminodiphenylsulphone, 4:4'-diaminodiphenylsulphoxide and 4-p-methoxybenzylidineamino-4'-aminodiphenylsulphone. None of these compounds produced continued inhibition of body growth (see Fig. 24). Inhibition occurred with all of the compounds for periods of 3–9 days after the beginning of treatment but subsequently growth at the normal rate occurred, although the compounds were administered at the same level. The loss of weight which occurred during the first few days of administration of the drugs may be due to increase in the excretion of urine, such as occurs when p-aminobenzenesulphonamide is given to rats [Rimington, 1938].

<div align="center">DISCUSSION</div>

Although treatment with prontosil soluble (Fig. 1) produced inhibition of tumour growth, the mice did not survive the treatment very long; red prontosil (Fig. 2) and p-aminobenzenesulphonamide (Fig. 3) which are equally effective as antibacterial compounds appeared to be more effective in inhibiting tumour growth and animals so treated survived much longer. Heptylideneaminobenzenesulphonamide (Fig. 4) and p-aminobenzenesulphonyl-p-sulphonamidophenylamide hydrochloride (Fig. 5) also have some action of this kind.

Buttle et al. [1936] found that sulphanilic acid has only about 1/20 of the therapeutical action of p-aminobenzenesulphonamide and that m-aminobenzenesulphonic acid and p-hydrazinobenzenesulphonic acid had only slight actions against streptococcal infections in mice. Tested on tumours m-aminobenzenesulphonic acid had no action (Fig. 7) while p-aminobenzenesulphonic acid (Fig. 8) had definite action and o-aminobenzenesulphonic acid (Fig. 6) and p-hydrazinobenzenesulphonic acid (Fig. 10) some slight action. The marked difference between the m- and p-aminobenzenesulphonates indicates that some specificity in tumour-inhibiting action may be found. Sodium sulphanilylsulphanilate (Fig. 9) which has been recommended for treatment of canine distemper [Dochez & Slanetz, 1938] was more effective than sulphanilic acid or p-aminobenzenesulphonamide.

The 4:4'-diaminodiphenyl- and 4:4'-dinitrodiphenyl-sulphides, -sulphoxides and -sulphones (see Figs. 11–16) all showed some action but the most effective compounds were the sulphoxides, particularly 4:4'-diaminodiphenylsulphoxide. Levaditi et al. [1937] claim that 4-nitro-4'-aminodiphenylsulphoxide is particularly efficacious against gonococcal infections. In the case of one mouse (Fig. 14, no. 426) treatment with 4:4'-diaminodiphenylsulphide appeared to increase the rate of growth.

The Schiff's bases 4-p-methoxybenzylidineamino-4'-aminodiphenylsulphone (Fig. 17) and 4:4'-diaminodiphenylsulphone-glucose anil (Fig. 18) did not appear to be as effective as the original sulphones. Two diphenylene sulphones. benzidinesulphone (Fig. 19) and tolidinesulphone (Fig. 20), produced definite inhibition, although in these compounds the amino groups are situated in the m-position to the sulphone group. It is hoped to be able to test other substances related to benzidinesulphone.

Tetradecamethylene-1:14-diaminedihydrochloride, which King et al. [1937] have shown to be effective against some trypanosome infections, had some inhibitory action (Fig. 21) as also had synthalin A and synthalin B (Figs. 22 and 23) which Jancsó & Jancsó [1935] found to have trypanocidal action in mice.

In those cases in which the mice survived and measurements were made after treatment had been stopped, the rate of growth of the tumours returned to normal indicating that the compounds did not produce a permanent inhibition
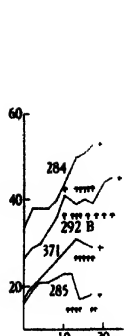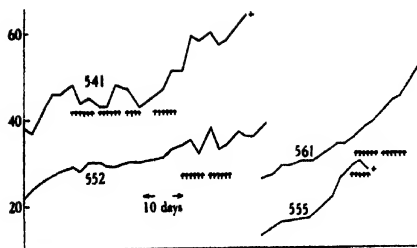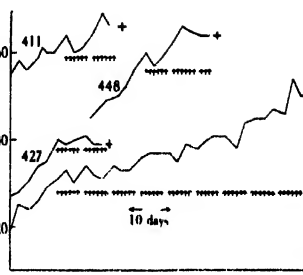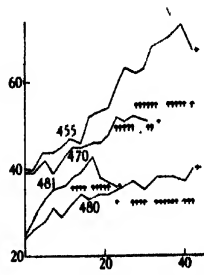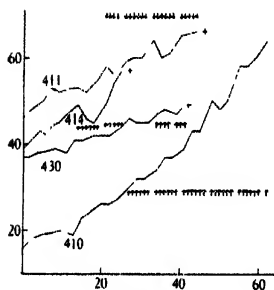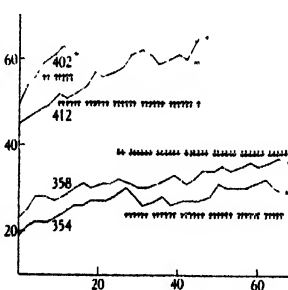
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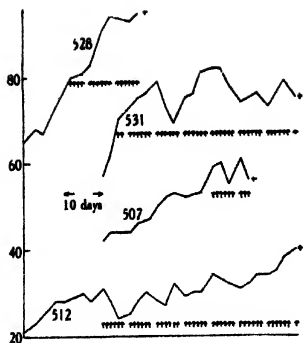
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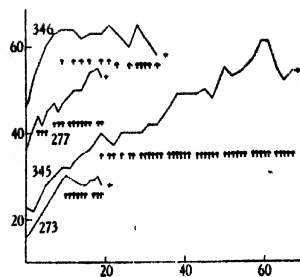
Fig. 3.

Fig. 4.

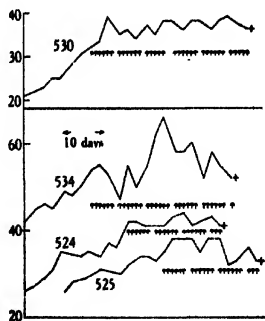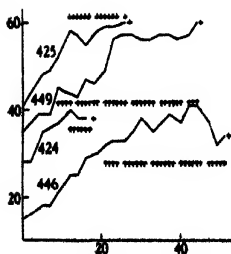Fig. 5.

Fig. 6.

Fig. 7.

Fig. 8.

Fig. 9.

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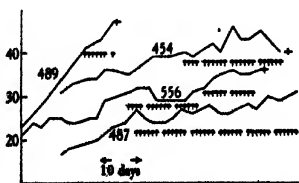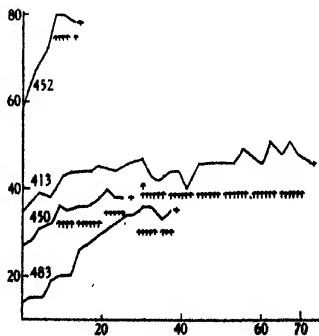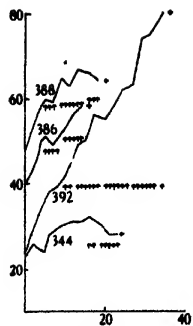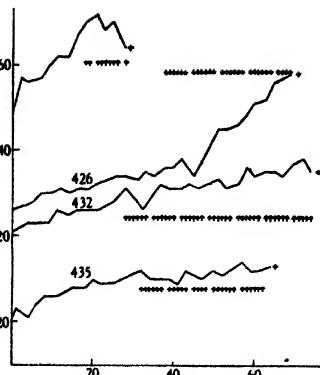
Fig. 11.
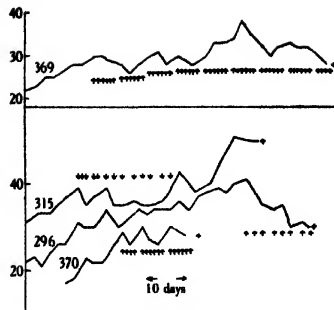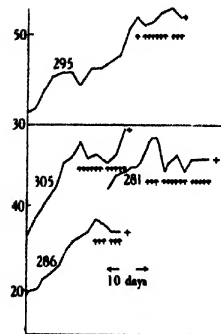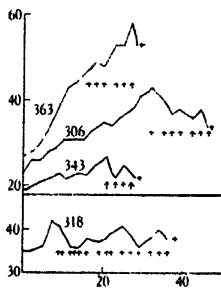
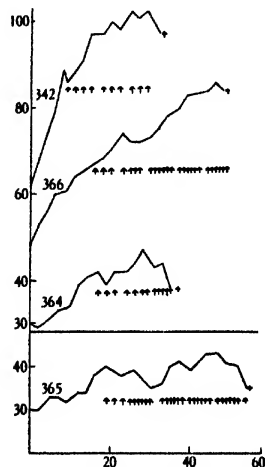Fig. 12.

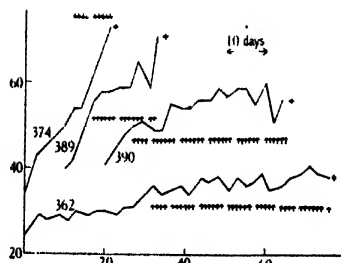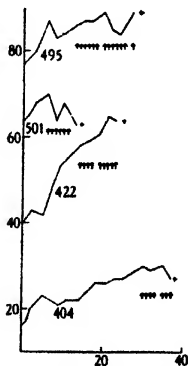For explanation of Figs. 1–12 see p. 1213.

Fig. 13.

Fig. 14.

Fig. 15.

Fig. 16.

Fig. 17.

Fig. 18.

Fig. 19.

Fig. 20.

Fig. 21

Fig. 22.

Fig. 23.

Fig. 24.

Control
4:4 diaminodiphenylsulphone
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4:4 diaminodiphenylsulphoxide
p aminobenzenesulphonamide
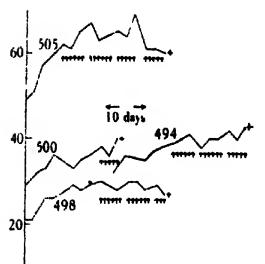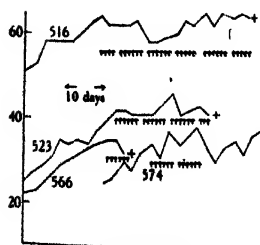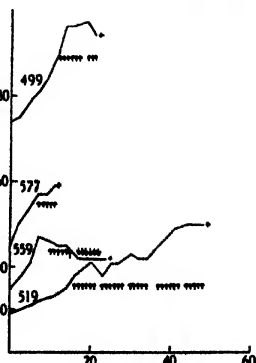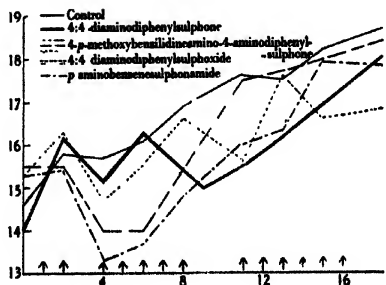
. For explanation of Figs. 13–24 see p. 1213.

of growth. This effect is to be seen in Fig. 4 (mouse 541) treated with *p*-heptylideneaminobenzenesulphonamide, Fig. 15 (mouse 315) with *pp'*-diaminodiphenylsulphoxide and Fig. 22 (mouse 574) treated with synthalin A.

The effect of these substances on growth in body weight (Fig. 24) and the fact that the growth of tumours is inhibited only while the drugs are administered indicate that the inhibition is somewhat different from that produced by carcinogenic compounds [Haddow *et al.* 1937]. Carcinogenic hydrocarbons generally exert a prolonged inhibitory action both on tumour growth and on growth of body weight.

Many of the treated tumours in which growth was inhibited became much softer. The degree of hardness of a tumour is difficult to assess but the softened tumours vary considerably in size from day to day. As a result of this, marked variations in tumour size from day to day are recorded as shown in Fig. 2, mice 507 and 531; Fig. 4, mouse 552; Fig. 5, mouse 434; Fig. 9, mice 525 and 534; Fig. 10, mouse 446; Fig. 12, mouse 413; Fig. 15, mouse 370; Fig. 21, mouse 281; Fig. 17, mouse 318; Fig. 23, mouse 342 and Fig. 19, mice 362 and 390.

From Table I it can be seen that of the compounds tested only 4:4'-diaminodiphenylsulphoxide and sodium sulphanilylsulphanilate inhibited the growth of the Crocker sarcoma, inhibited the growth of all spontaneous tumours used and caused retrogression in the case of three out of four of the mice with spontaneous tumours. Other compounds related to these are being investigated.

SUMMARY

Some aromatic sulphur compounds, have been found to retard the growth of spontaneous mammary cancer in mice. The most effective of the compounds tested appeared to be *pp'*-diaminodiphenylsulphoxide and sodium sulphanilylsulphanilate.

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EXPLANATION OF FIGS. 1–24.

Abscissae: Figs. 1–24: Days.

Ordinates: Figs. 1–23: Tumour size as sum of length and breadth in mm.
Fig. 24: Average wt. of mice in g.

Fig. 1. Growth of spontaneous mammary tumours in mice treated with 7-acetamino-2:4′-sulphonamidobenzene-azo-1-naphthol-3:6-disulphonate (prontosil soluble).

In this and other figures each arrow represents the administration of the dose shown in Table 1.

Fig. 2. Growth of mammary tumours in mice treated with 4-sulphonamidobenzene-azo-2′:4′-diaminobenzene (red prontosil).

Fig. 3. Growth of mammary tumours in mice treated with p-aminobenzenesulphonamide.

Fig. 4. Growth of mammary tumours in mice treated with p-heptylideneaminobenzenesulphonamide.

Fig. 5. Growth of mammary tumours in mice treated with p-aminobenzenesulphonyl-p′-sulphonamidophenylamide hydrochloride.

Fig. 6. Growth of mammary tumours in mice treated with sodium o-aminobenzenesulphonate.

Fig. 7. Growth of mammary tumours in mice treated with sodium m-aminobenzenesulphonate.

Fig. 8. Growth of mammary tumours in mice treated with sodium p-aminobenzenesulphonate.

Fig. 9. Growth of mammary tumours in mice treated with sodium sulphanilylsulphanilate.

Fig. 10. Growth of mammary tumours in mice treated with sodium p-hydrazinobenzenesulphonate.

Fig. 11. Growth of mammary tumours in mice treated with 4:4′-dinitrodiphenylsulphide.

Fig. 12. Growth of mammary tumours in mice treated with 4:4′-dinitrodiphenylsulphoxide.

Fig 13. Growth of mammary tumours in mice treated with 4:4′-dinitrodiphenylsulphone.

Fig. 14. Growth of mammary tumours in mice treated with 4:4′-diaminodiphenylsulphide.

Fig. 15. Growth of mammary tumours in mice treated with 4:4′-diaminodiphenylsulphoxide.

Fig. 16. Growth of mammary tumours in mice treated with 4:4′-diaminodiphenylsulphone.

Fig. 17. Growth of mammary tumours in mice treated with 4-p-methoxybenzylideneamino-4′-aminodiphenylsulphone.

Fig. 18. Growth of mammary tumours in mice treated with 4:4′-diaminodiphenylsulphone glucose anil.

Fig. 19. Growth of mammary tumours in mice treated with benzidinesulphone.

Fig. 20. Growth of mammary tumours in mice treated with tolidinesulphone.

Fig. 21. Growth of mammary tumours in mice treated with tetradecamethylene-1:14-diamine-dihydrochloride.

Fig. 22. Growth of mammary tumours in mice treated with decamethylenediguanidine hydrochloride (synthalin A).

Fig. 23. Growth of mammary tumours in mice treated with dodecamethylenediguanidine hydrochloride (synthalin B).

Fig. 24. The growth in body weight of mice treated with certain sulphur compounds.

CLXIII. FAT METABOLISM IN FISHES
XIII. FACTORS INFLUENCING THE COMPOSITION OF THE DEPOT FAT OF FISHES

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THERE are at least four factors which possibly affect the composition of the depot fat of a fish. These are (1) species, (2) diet, (3) and (4) temperature and salinity of the water.

Species. It is well known that different species of animals lay down depot fats more or less characteristic of that animal. With normal diet the composition of the depot fat will remain roughly the same. In the case of fish the author [Lovern, 1937] has shown that various characteristic depot fats are produced from a roughly uniform diet, according to species, their composition thus being largely independent of diet.

Diet. It is equally well known that the diet of an animal may affect the composition of its depot fat. The process, however, is by no means quantitative, and when it is studied in detail some contradictory results are obtained. There are two types of experiment which throw light on this problem.

In the first type certain fatty acids are fed which can be detected if they are incorporated in the depot fat, e.g. elaidic acid and acids containing deuterium. Schoenheimer & Rittenberg [1935] found that in mice fed for 4 days on a diet containing 1 % of fat containing deuterium (the only fat in the diet), 47 % of the ingested fat was found in the depots. The authors conclude that the largest part of the dietary fat, even at this low level of ingestion, is deposited in the fatty tissues before it is used. In another experiment the same workers [Rittenberg & Schoenheimer, 1937] find evidence of a continual turnover between depot fat and ingested fat and suggest a half-lifetime for depot fatty acids of 5–9 days.

In the second type of experiment fats are fed which differ from the normal depot fat of the animal in the proportions of the different acids present, or in the unsaturation of some of these. Here the results obtained are quite different and sometimes contradictory. Thus, Thomas *et al.* [1934] found that liberal allowances of whole soybeans, menhaden oil, corn oil and coconut oil fed to steer calves for 260 days had no perceptible effect on the unsaturation of the body fats. On the other hand, Ellis [1933] found a marked decrease in saturation in pig's fat after feeding peanuts and an increase in saturation after feeding corn. Possibly the different species of animal used has something to do with this apparent contradiction. It should also be remembered that whilst a change in iodine value indicates a change in composition, a constant iodine value does not necessarily connote a constant composition.

The experiments of this second type uniformly indicate that fat must be fed at a fairly high level before any considerable effect is produced on the body fat. Thus, Banks & Hilditch [1932] found 1–2 % of C_{20} and C_{22} unsaturated acids in the fat of a pig fed on a diet containing 0·7 % of fish fat. Brown [1931] fed pigs on a diet containing 14 % of menhaden oil, but the content of C_{20} and C_{22}

unsaturated acids in the fat was only 2·7 %, so that all this extra fish fat had had little effect on the depot fat. Bhattacharya & Hilditch [1931] showed that 3 % of shea butter or of arachis oil in the diet of a pig made little difference to the proportions of the various acid groups, and suggested that acids ingested in excess are disposed of (by oxidation) to leave a residue "more or less adapted to the requirements of the particular storage fat". Again, Banks *et al.* [1933] found that when about 5 % of cod liver oil was included in the diet of rats, there was not much alteration in the depot fat, apart from some deposition (about 8 %) of C_{20} and C_{22} acids. The rest of the acids appeared to be present in much the same relative proportions as when the diet was mainly fat-free. Therefore the ingested cod liver oil had not been freely incorporated in the rat's depot fat.

The discrepancy between the results obtained by experiments of these two types may be understandable. There are two characteristics of a fatty acid which may affect a fat in which it is incorporated—unsaturation and length of carbon chain. It may be that the requirements of an animal with regard to the chain lengths of its depot fatty acids are specific. In the case of fish, where there is a great range of chain lengths amongst the various fatty acids, the relative proportions of these groups of acids are often quite characteristic of the species. Now a fatty acid containing deuterium, for instance, might well become incorporated in the depots in place of some normal acid of the same chain length without upsetting the animal's specific requirements. Whatever the reason, there seems little doubt that whilst normally there is constant interchange between ingested fat and depot fat (as shown by the deuterium experiments), this is not unconditionally the case when a fat with unusual proportions of the various acids is fed in the diet. Some selective mechanism then operates, and surplus ingested acids of various chain lengths are eliminated or modified until a mixture resembling the normal depot fat remains. If, however, the unusual fat fed is above a certain limit this selective mechanism partly breaks down.

There has been very little work done on the effect of different dietary fats on fish.

Temperature. It is generally accepted that temperature has an effect on the composition of the fats of both animals and plants, higher temperatures tending to produce more saturated fats. It should be noted that this only applies to a given species under varying conditions of temperature. This effect has been studied, for example, in pigs, and Dean & Hilditch [1933] have confirmed and amplified the earlier results of Henriques & Hansen [1901]. In the case of fish, Brocklesby & Bailey [1932] found an increasing iodine value in the body oil of the salmon as they went further north (provided that they kept to the same species of salmon), and stated that this corroborated the findings of investigators working on other species of fish. Tropical fish do not necessarily have a more saturated fat than other (even closely related) species inhabiting colder waters [Lovern, 1932; 1935, 2].

Salinity. It has been shown repeatedly [Lovern, 1937] that fresh-water fish have fat different from that of marine fish, but the reason has not yet been found. The difference is also found in the vegetable-feeding zooplankton [Lovern, 1935, 1]. These small Crustacea must produce these characteristic types of fat by appropriate modification of ingested fat, at least in the case of the marine organisms [Lovern, 1936]. Whilst this effect is apparently associated with salinity alone, it may be an indirect effect. Thus, Fox & Simmonds [1932] have found that the oxygen uptake of Crustacea is greater in fresh water than in salt water, and the whole metabolic rate is higher.

In the experiments described in this paper an attempt has been made to study the individual effects of diet, temperature and salinity on the fat of a suitable species of fish. The common eel, *Anguilla vulgaris*, was chosen because (a) it has a high fat content and adequate quantities of material can be obtained from a reasonable number of individuals, and (b) it can live in fresh water or sea water.

EXPERIMENTAL

The apparatus consisted of three large tanks, each of which was connected to a smaller tank. The small tanks, placed below the larger ones, were fitted up as thermostats with both heating and cooling appliances. Water ran continually through gravel filters from the upper into the lower tanks, and was pumped back as an aerated froth. The eels were confined in the large tanks. They were fed daily, and each day the water was changed, affording an opportunity for cleaning the tanks and removing unconsumed food. Refilling was done slowly through the thermostatic tanks to minimize temperature disturbances.

In the first experiment the tanks were set up as follows: 1, warm sea water; 2, warm fresh water; 3, cold fresh water. Each tank was stocked with 50 eels 30–35 cm. in length. Until feeding had commenced the temperatures were maintained at about 15°. In fresh water the eels refused food for about 10 days, but in sea water they commenced feeding at once. The diet consisted of shelled mussels (*Mytilus edulis*) and was fed *ad lib*. After feeding had become general, the temperatures were adjusted to the highest and lowest limits at which feeding was satisfactory. On raising the temperature progressively from 15°, the daily consumption of food increased until a temperature of 23° was attained. After this point consumption declined, and so this temperature was maintained for the two warm-water tanks. Similarly, on lowering the temperature from 15° feeding was diminished, and below 12° almost ceased. A temperature of 14° was selected as the most suitable for the cold-water tank and was maintained throughout the experiment.

Unfortunately, in this first experiment, no record was kept of increases in weight or of food consumption. Each month a sample of mussels was examined for fat content; there was little variation, the total ether-soluble matter averaging about 1·35 %. Feeding was continued for 6½ months, when the eels were killed and the body fats extracted. In all the tanks there were a few deaths due to initial injury and to fighting, and in two cases (after 3 months in warm sea water and 5 months in warm fresh water) there was a breakdown of aeration which caused the deaths of about 20 eels in each case, the remainder in each tank recovering. These latter dead eels were also extracted and their body fats examined.

As stated, the eels were originally all about 30–35 cm. in length. It is necessary to have a uniform length to get roughly uniform fat content. The fat content of the eel increases almost linearly with length, at any rate between certain limits (Fig. 1). Thus, on the average, these eels would start with about 9 % of fat.

At the end of the experiment some individuals appeared to have grown appreciably, whilst others had not. It is known that the so-called "male" eels do not grow so fast as the female eels, and possibly the differences observed were due to mixed "male" and female eels being used. There was a greater preponderance of larger eels in tank 3 at the end of the experiment than in the other tanks.

The fat contents of the eels are given in Table I and the composition of the fats in Table II. A control sample of eels of the same length obtained from the same source (estuarine waters of the River Dee), is included in both tables.
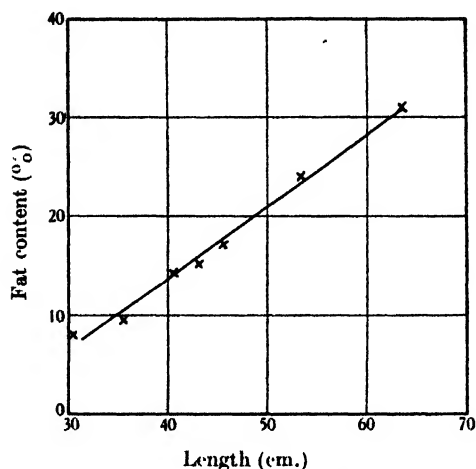
Fig. 1. Fat content of eels.

Table I. *Fat contents of eels*

Batch no.	Description	Period fed months	Fat content %	Iodine value
1	Control	—	9·3	118·5
2	Fresh water 14"	6½	18·0	119·1
3	,, 23"	5	8·2	126·4
4	,, 23	6½	10·2	121·5
5	Sea water 23'	3	8·9	118·5
6	,, 23"	6½	9·4	116·6

Table II. *Composition of fatty acids of eel fats (wt. %)*

Batch no.	Saturated			Unsaturated				
	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
1	4·3	16·8	2·5	0·1	8·8 (−2·2 H)	39·4 (−2·5 H)	20·8 (−5·6 H)	7·3 (−10·2 H)
2	3·3	20·5	2·7	Nil	11·6 (−2·4 H)	39·4 (−2·7 H)	16·1 (−6·3 H)	6·4 (−10·0 H)
3	3·6	17·9	2·8	Trace	6·8 (−2·3 H)	39·2 (−2·6 H)	22·4 (−5·8 H)	7·3 (−8·0 H)
4	4·1	19·4	2·2	Nil	5·7 (−2·1 H)	37·5 (−2·8 H)	20·6 (−5·5 H)	10·5 (−9·2 H)
5	3·1	19·8	4·8	0·7	5·8 (−2·4 H)	42·6 (−2·7 H)	15·7 (−5·5 H)	7·5 (−8·0 H)
6	3·7	17·4	3·9	Nil	9·5 (−2·1 H)	39·6 (−2·4 H)	16·8 (−5·2 H)	9·1 (−8·6 H)

A large quantity of mussels was extracted, and the ether-extract (123 g.) separated into acetone-soluble fat (73 g.) and acetone-insoluble crude phosphatides (50 g.). The mixture of fatty acids recovered from each fraction was analysed. These mussels were obtained in December 1936. In October 1937 (in connexion with a second experiment) another, larger batch of mussels was extracted, giving 324 g. acetone-soluble fat and 152 g. phosphatides. The original analysis on the fat fraction was satisfactory, but the analysis of phosphatides

was deemed unreliable, so another analysis was carried out on the second batch of phosphatides. In each case appreciable amounts of organic acids of non-fatty nature were encountered, also about 20 % of highly unsaturated acids of high molecular weight (about C_{26}?). Both phosphatide acid analyses are only approximate in view of the considerable experimental difficulties. These unusual acids did not occur in the acetone-soluble fat. The figures for the mussel lipoids are given in Table III (with non-fatty acids and C_{26} (?) acids excluded).

Table III. *Fatty acids of mussel lipoids (wt. °/₀)*

Material	Saturated				Unsaturated				
	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}	C_{24}
Fat	1·9	16·7	1·7	0·3	10·6	21·5	29·9	13·9	3·5
					(−2·5 H)	(−4·1 H)	(−7·3 H)	(−9·3 H)	(−? H)
Phosphatide (1)*	Nil	28·5	14·4	Nil	3·2	10·1	29·9	13·2	?
					(−3·5 H)	(−4·6 H)	(−6·4 H)	(−8·6 H)	—
Phosphatide (2)	Nil	27·3	6·2	Nil	5·0	16·4	32·2	12·9	?
					(−3·2 H)	(−4·0 H)	(−6·0 H)	(−8·5 H)	—

* Plus 0·7 % arachidic acid.

This first experiment was considered unsatisfactory in several respects—in particular, the lack of records of growth and food consumption, and the use of mixed "male" and female eels with different growth rates. A second experiment was undertaken with the conditions as follows.

(1) The tanks were filled with (a) sea water at 14°, (b) fresh water at 14°, (c) fresh water at 23°.

(2) Each tank was stocked with 25 eels 50–55 cm. in length. This is above the supposed maximum size for "male" eels.

(3) The combined weight of eels in each tank was taken at the start and finish of the experiment.

(4) The diet in the fresh-water tanks consisted of herring. A large supply of herring in uniform condition was obtained at the start of the experiment. This was kept in cold storage and the required amount withdrawn daily. It was fed as pieces of flesh (with skin) about 2 cm. square, containing 20·7 % fat. The diet in the sea-water tank consisted of mussels.

(5) The food was fed *ad lib.*, and the daily consumption was measured.

(6) Any eels which died during the course of the experiment (which ran for 6 months) were weighed and the weight was subtracted from the initial weight. It was assumed that they had eaten nothing since the commencement of the experiment, which was in most cases probably true, owing to initial injuries.

The particulars of the eels are given in Table IV and the composition of their fat in Table V. A control sample of eels of 50–55 cm. length, obtained from the estuary of the Dee, was also examined. The composition of the fat of the herring used is included in Table V. The samples are numbered as follows: 1, control; 2, sea water at 14°; 3, fresh water at 14°; 4, fresh water at 23°. The initial weights are the corrected ones, allowing for 10 deaths in sample 2, 7 deaths in sample 3, and 8 deaths in sample 4.

Table IV. *Particulars of eels in Exp. 2*

Sample	Initial wt. g.	Final wt. g.	Total food eaten ·g·	Final fat content %	Iodine value
1	—	—	—	24·1	119·0
2	3038	2574	10,459	16·6	114·1
3	2732	3159	4,140	20·6	140·4
4	2829	3556	5,739	23·9	137·6

Table V. *Fatty acid compositions of eel fats in Exp. 2 (wt. %)*

Sample	Saturated				Unsaturated			
	C_{14}	C_{16}	C_{18}	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
1	4·3	17·8	1·7	Trace	9·2	38·4	20·1	8·5
					(−2·2 H)	(−2·7 H)	(−6·0 H)	(−9·3 H)
2	4·8	17·6	2·6	—	9·4	41·6	16·4	7·6
					(−2·2 H)	(−2·4 H)	(−5·8 H)	(−9·1 H)
3	6·4	17·1	1·3	—	6·9	31·9	22·2	14·2
					(−2·4 H)	(−3·0 H)	(−5·4 H)	(−7·5 H)
4	6·0	16·5	1·1	0·6	8·3	33·9	22·6	11·0
					(−2·2 H)	(−2·8 H)	(−5·5 H)	(−7·0 H)
Herring	8·3	12·1	0·3	0·5	6·4	21·0	28·3	23·1
					(−3·4 H)	(−4·5 H)	(−5·5 H)	(−4·6 H)

The eels in the cold sea water were fed on mussels to obtain data on increase (or loss) in weight on a diet so low in fat. In Exp. 1 there was reason to suspect considerable deposition of fat in the cold fresh water, judging by the unusually high fat content (18 %) of the eels. This was the more remarkable since the eels in cold water eat less of any given food than those in warm water. Actually, there was a loss in weight (as well as a reduction in fat content) on a similar diet in cold sea water (Table IV).

An attempt was made to keep eels in sea water on a diet of herring in a smaller tank than the three described. The water was not circulated, but was well aerated and changed daily. For some reason the eels refused to eat anything but particularly tempting food, such as live earthworms. After 5 months' total starvation the attempt was abandoned.

DISCUSSION

It will, perhaps, be best if the influence of the various factors, diet, temperature and salinity, are discussed under separate headings, although some overlapping is inevitable.

Diet. The diets fed, mussels and herring, probably represent about the extremes of non-fatty and fatty food likely to be eaten by an eel in its natural environment. The mussels contained on an average 1·35 % of ether-soluble matter. Allowing for the 15 % of unsaponifiable matter present in this and for the lower proportion of fatty acids in phosphatides than in true fats, the mussels contain about 1·1 % of fat. Not many aquatic organisms will have appreciably less fat than this, and not many will have more than the 20·7 % of fat present in the herring.

Both the dietary fats differ considerably in composition from eel's fat. In the mussel, considerable proportions of phosphatide are present. The acids of the neutral fat and of the phosphatides differ mainly in the presence of more palmitic and stearic acids and less C_{16} and C_{18} unsaturated acids in the phosphatides. If total groups are considered (all C_{16} acids, for instance, being considered together), there is no marked difference between the fatty acid compositions of the three materials included in Table III. Indeed, one could imagine that the glycerides and phosphatides of the mussel had been formed from almost the same mixture of acids, with subsequent selective hydrogenation of the phosphatide mixture. The main differences between the mussel's fatty acids and those of the eel (no. 1 of Table II and also of Table V are very similar) are in respect of the presence of considerably less C_{18} acids, less C_{14} and more C_{20} and

C_{22} acids in the mussel. The most marked differences between herring's fat and eel's fat are in respect of the presence of much more C_{22} acids and much less C_{18} acids in the former. All the other groups, however, are appreciably different in the two fats, and there are more C_{14} and C_{20} and less C_{16} (especially total C_{16}) acids in the herring's fat.

The depot fat of the eels should have been appreciably modified if there had been any considerable incorporation of the ingested fat. From Table II, and also from comparison of samples 1 and 2 in Table V, it can be seen that there was no tendency for the fat of eels on a diet of mussels to be modified in the direction of mussel's fat. This is in accordance with evidence already quoted, that dietary fats differing from the fat of the experimental animal in the relative proportions of the various acids must be fed·above a certain level before modification takes place.

On the diet of herring there has been appreciable modification of the eel's fat in the direction of herring's fat (Table V, samples 1, 3 and 4). In fact, the final compositions attained in samples 3 and 4 correspond well with those of definite mixtures of eel's fat and herring's fat. In Table VI are given the calculated compositions of mixtures of 62 % eel's fat with 38 % herring's fat and 78 % eel's fat with 22 % herring's fat, expressed as total groups, compared with the actual fats of samples 3 and 4 (Table V) respectively. The analytical method used is not sufficiently accurate, of course, to differentiate between, say, a 60/40 mixture and a 62/38 mixture. Furthermore, the original eel's fat and the ingested herring's fat were probably not absolutely uniform in composition.

Table VI. *Mixtures of eel and herring fats*

Material	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}
62/38 mixture	6·0 (+)	23·8	33·0	23·2	14·0
Sample 3	6·4	24·0	33·2	22·2	14·2
78/22 mixture	5·3	25·1	36·0	21·9	11·7
Sample 4	6·6	24·8	35·0	22·6	11·0

It is evident from Table VI that when the ingested fat does find its way into the fat depots of the eel, it does so with the relative proportions of its various acids practically unaltered. Schoenheimer & Rittenberg's [1935] experiments suggest a deposition of most of the ingested fat in the depots before use, and other experiments [Rittenberg & Schoenheimer, 1937] suggest a continual turnover between depot fat and ingested fat. Whilst this may be true for normal dietary fats, it cannot be unconditionally so for dietary fats differing appreciably from the depot fat of the animal. The results with the eel demonstrate this. On the theory of continual turnover it is possible to calculate the final composition of the eel's fat by assuming that all the ingested fat is deposited in the depots and then a certain amount of the mixed fat is withdrawn.

Errors in the calculation may be caused by the assumption that all the eels in Exp. 2 started with about 24 % of fat. Fig. 1 was compiled from results obtained on batches of about 20 eels of each size. As the experimental batches consisted of a fair number of eels in each case, there is a good chance of individual variations being largely compensated. Moreover, it is unlikely that the average of all three batches varied much from 24 %. For the sake of the following calculations a uniform initial fat content of 24 % in Exp. 2 will be assumed.

From Table IV we can calculate the daily intake of fat and the daily retention. It will not cause serious error to assume a constant daily intake of 1/180th of the total amount eaten. On this basis sample 2 consumed 0·64 g. of mussel's

fat daily, and lost 301·4 g. of their own initial fat, or a total daily loss of 2·31 g. If this 0·64 g. was deposited unchanged in the depots and 2·31 g. of the mixed fat was withdrawn, the final composition after 180 days should be 81 % eel's fat, and 19 % mussel's fat. Such a mixture does not differ from eel's fat sufficiently to be detectable by analysis. Actually, the fat of sample 2 differs slightly from that of sample 1 in the reverse direction from that produced by admixture of mussel's fat with eel's fat. This is presumably due to variability in eel's fat itself and to experimental error. The apparent failure of a diet low in fat to affect the composition of the depot fat, may, therefore, in this instance at least, be due to the limitations of the analytical procedure.

Sample 3 ingested 4·76 g. of herring's fat daily and the daily loss of fat was 4·78 g. By the above hypothesis this process would finally give a mixture of 28 % eel's fat and 72 % herring's fat, contrasted with the 62 % eel's fat: 38 % herring's fat mixture actually found. Again, in sample 4 there was a daily ingestion of 6·60 g. of herring's fat and a daily loss of 5·65 g., which should ultimately give 24 % eel's fat and 76 % herring's fat, contrasted with the 78 % eel's fat: 22 % herring's fat mixture found. Such differences could readily be detected by analysis.

The differences are so large that it seems improbable that they can be attributed to error in the assumption of an initial fat content of 24 %. An initial fat content of 20 %, for instance, gives final compositions of the same order as with the 24 % figure. The absorption of the mussel's and herring's fats by the eels should be almost complete. McCay & Tunison [1934], in experiments on trout, showed good absorption of various fats fed even at 25 % of the diet. The conclusion of the writer is that there cannot have been a quantitative turnover between ingested fat and depot fat.

The hypothesis of Schoenheimer & Rittenberg [1935] may be open to question. If admixture with the fat already in the depots is thorough before withdrawal of mixed fat for combustion, then in the case of mice fed at the 1 % level for 4 days one would expect to find about 95 % of the dietary fat in the depots instead of the 47 % actually found. It is possible, of course, that the incorporation of ingested fat in the depots is not uniform, and that the recently deposited fat is more readily withdrawn than the rest of the depot fat. Either of these conditions (i.e. partial deposition only or uneven admixture and withdrawal), or perhaps both together, could account for the observed results on eels not being fully quantitative.

An interesting point is the high fat content of eels in batch 2, Table I. Although no records of weight were taken, there was visible evidence of growth, and this on a diet of mussels. The reason is obscure, but it may be noted that the final fat showed no evidence of having been modified by the diet. The eels feeding on mussels in Exp. 2 failed to maintain weight.

Although the idea of a complete interchange between ingested fat and depot fat is not in harmony with the evidence, neither is the idea that there has been no interchange, and that the only herring's fat deposited is that represented by the total gain in fat on the diet of herring. On this basis the final fat of sample 3 (second experiment) should have consisted of entirely unchanged eel's fat.

Evidently the quantitative effect of the dietary fat on the depot fat is governed by factors which are still obscure. The metabolic rate appears to have something to do with it, since the warm-water fish, in spite of ingesting more herring's fat than did the cold-water fish, did not modify their fat so much.

Temperature. In considering the effects of temperature we should not compare various samples with the control estuarine sample, since the temperature

of the estuary was variable and unknown. In Table II we can compare sample 2 with samples 3 and 4, where the only difference was one of 9° in temperature. There is a slight suggestion that the fat in cold water is more unsaturated (certainly not shown by the iodine value, however, as seen from Table I). C_{16}, C_{20} and C_{22} unsaturated acids are slightly more unsaturated in sample 2, whilst C_{18} acids are about the same. There is evidence of the production of a more saturated fat at higher temperatures, but the effect is certainly not a large one.

In the second experiment we may compare samples 3 and 4, with similar results. C_{16}, C_{18} and C_{22} acids are slightly more unsaturated in the cold water than in the warm water, C_{20} acids being about the same. In this experiment, however, the position is complicated by the different amounts of herring's fat which have been incorporated in the depots. Herring's fat is more unsaturated than eel's fat for C_{16} and C_{18} acids, and less unsaturated for C_{22} acids. C_{20} acids are about the same (if both samples of estuarine eel's fat are considered). The fact that less herring's fat has been incorporated in the fat of the eels in sample 5 than in that of the eels of sample 4 would mean that the results for C_{16} and C_{18} acids could be accounted for on this basis. The results for C_{22} acids could not, however, be so interpreted and so here the evidence supports that of Exp. 1.

Actually, whilst as far as total groups are concerned the herring's fat appears to be deposited with the relative proportions unchanged, the degrees of average unsaturation show distinct changes. In Table VII are given the degrees of average unsaturation of C_{16}, C_{18} and C_{22} unsaturated acid groups.

Table VII. *Degrees of unsaturation of eel fats*

Material	Degrees of unsaturation		
	C_{16}	C_{18}	C_{22}
62% eel fat 38% herring fat	− 2·7 H	− 3·3 H	− 7·5 H
Sample 3	− 2·4 H	− 3·0 H	− 7·5 H
78% eel fat 22% herring fat	− 2·5 H	− 3·2 H	− 8·5 H
Sample 4	− 2·2 H	− 2·8 H	− 7·0 H

It can be seen from Table VII that the eels in both warm and cold water have partially hydrogenated either the herring's fat itself or more probably the mixed fat in the depots. In the cold water such hydrogenation has been confined to the C_{16} and C_{18} acids—the ones which the diet would tend to make more unsaturated. However, in the warm water the C_{22} acids are also hydrogenated, and this difference is presumably due to the higher temperature. The extent to which the other acids are hydrogenated is almost the same in the two samples.

Both experiments, therefore, afford evidence of greater unsaturation in fats laid down at lower temperatures, but the effect is certainly not a marked one. It is notable that whilst in both samples 3 and 4 hydrogenation has gone far enough to bring the unsaturation of the C_{16} and C_{18} acids almost to normal (Table V), there is not hydrogenation to such an extent that the iodine value of the entire fat is normal (Table IV). This is a further illustration of the comparative unimportance of the iodine value as a guide to metabolic processes in fats.

The principal effect of a higher temperature in Exp. 2 has been to accelerate the process of modifying ingested fat.

Salinity. From the results of Exp. 1 it is evident that under such conditions salinity has had no effect. Comparing samples 5 and 6 with 3 and 4 (Table II), there is no evidence at all of a tendency towards a marine type of fat in sea water or a more truly fresh-water type in the fresh water. The differences between these types have been illustrated in detail [Lovern, 1937], and consist of higher proportions of C_{16} and especially C_{18} acids, and lower proportions of C_{20} and especially C_{22} acids, in the fats of fresh-water fish. The estuarine eels' fats (controls) correspond fairly well to the fresh-water type, but are not really typical—in particular the C_{16} acids content is on the low side and the C_{20} acids content on the high side. The reason may be that they were obtained from brackish estuarine waters. That such a habitat may lead to a modified fat was suggested by the author [Lovern, 1937]. Shorland & McIntosh [1936] found that a closely related eel from New Zealand had fat resembling that of typical fresh-water fish.

It should perhaps be noted that in these experiments aeration would probably not be as complete as under natural conditions. If the differences in metabolic rate observed by Fox & Simmonds [1932] are due to the greater oxygen content of fresh water compared with sea water (as they suggest), such differences might not obtain in the experimental tanks.

It seems doubtful whether any fat was laid down under the conditions of Exp. 1 (apart from batch 2). It might be argued that the failure of salinity to have any effect was due to this, and that only fat actually deposited whilst the fish are in a certain environment can be affected by that environment. It has been shown [Lovern, 1934] that starving salmon in fresh water do not modify their fat in the direction of the fresh-water type. These eels were not starving, however, and unless the depot fat remained in a static condition with no ebb and flow (and the experiments on feeding herring definitely indicate an ebb and flow), it seems hard to avoid the conclusion that salinity has little if any direct effect.

The experiments with a diet of herring are difficult to interpret. Herring's fat is markedly marine in type, and yet 6 months' feeding on this fat at as high a level as is ever likely to be encountered in nature, did not make the eel's fat truly marine in type. It is notable that the more intensive feeding in the warm water, so far from causing the fat to become still more marine, actually resulted in less change. This suggests that there is in the animal a mechanism which can deal with ingested fat so as to modify it to the normal requirements of its depot fat. This mechanism might be unable to deal with fat above a certain level of ingestion, but its activity might well be increased at higher temperatures.

It is impossible to tell from these experiments how far it would be possible to modify the fat of a fish by an unusual diet if that diet was fed throughout its life. On the other hand, the existence of a mechanism which can modify ingested fat to a certain extent, and the fact that the rate at which this mechanism functions can be altered (e.g. by changes of temperature) is significant. Such a mechanism, with a variable rate, has been adduced for the herring [Lovern, 1938].

SUMMARY

Using eels (*Anguilla vulgaris*) as experimental animals, an attempt has been made to determine the separate effects of diet, temperature and salinity on the composition of the depot fats of fish.

On a diet low in fat ($1 \cdot 1 \%$), the ingested fat had no detectable effect on the depot fat. On a diet high in fat ($20 \cdot 7 \%$), the eel's depot fat was appreciably modified, but the quantitative relationship between the amount ingested and the effect produced was obscure. Such ingested fat as was incorporated in the depot

fat was apparently deposited with the relative proportions of the various acids practically unchanged. Hydrogenation, however, had taken place. The eel possesses a mechanism which can modify ingested fat to a considerable extent, and the action of this mechanism is accelerated by rise of temperature.

In addition to the above property, temperature has a direct effect on the composition of fat. Lower temperatures lead to more unsaturated fat and higher temperatures to increased saturation. This effect is relatively a small one, and in natural conditions cannot be important.

The effect of salinity has not been thoroughly settled, but with a low intake of fat it appears to have no direct significance.

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CLXIV. ON THE RESPIRATORY METABOLISM OF *HELIX POMATIA*

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THE results brought forward in this paper have been accumulated during the last three years in the course of experiments on the respiratory metabolism of the edible snail, *Helix pomatia*. This paper does not purport to give a complete picture of the mechanisms concerned in the metabolism of this animal, but rather to summarize the situation as it appears at present, to indicate some metabolic peculiarities which have come to light and to draw attention to some interesting problems which still await solution. The work arose out of an attempt to obtain some information as to the general metabolism of carbohydrates in *Helix*, and it seemed worth while to extend the experiments to cover some other substrates of oxidation.

MATERIAL AND METHODS

The tissue used was the so-called "liver" or hepatopancreas of *Helix pomatia*, an organ which, according to Biedermann [1911], is responsible for the digestion, absorption and storage of the food. There can be little doubt of the metabolic importance of this organ. It is a large, dark brown structure which accounts for the greater part of the whole visceral mass, and produces numerous enzymes, including cellulase, α- and β-glucosidases, α- and β-galactosidases, fructosaccharase and other but less well-defined carbohydrases such as lichenase [see Krüger, 1933; Vonk, 1937]. Proteinases and aminopolypeptidase [Rosèn, 1934] and one or more lipases [Kuntara, 1934] have also been found either in the secretion or in the substance of the gland itself.

The main stock of animals was kept in the refrigerator and samples of a dozen or so were taken out often enough to keep up a supply of experimental animals. The use of manometric technique and tissue slices were the principal methods employed, the directions of Dixon [1934] being followed with only minor alterations. Slices were cut from the hepatopancreas of animals which had been starved for 2 to 4 weeks at room temperature; this preliminary starvation went far towards removing stored foodstuffs and made it possible to cut thin slices from the tissue which, when full of stored materials, has the consistency of a soft rubber sponge and is exceedingly difficult to manipulate. The slices were washed in the phosphate-buffered saline described below (agitated by an air blast) till the washings were no longer turbid. No definite indications of a loss of coenzymes or carriers were encountered at any stage in this work.

After the slices were freed from surplus fluid by draining on filter paper, they were weighed on the torsion balance before being placed in the manometric flasks. The dry wt. was determined on a separate small sample of the same tissue when sufficient material was available. In all, 47 such determinations were carried out, the results varying from 14 to 22% and averaging 18% of the wet wt. This value was assumed in cases where a separate estimation of the dry wt. was not made.

The solution used for washing and suspending the tissue slices had the composition given below. The various substances the effect of which was to be tested were added to the stock solution, usually to give a final concentration of $M/100$:

	g./l.
$Na_2HPO_4, 12H_2O$	16·120
NaH_2PO_4, H_2O	0·690
NaCl	0·141
KCl	0·443

This solution is allowed to stand for at least 3 days before use since, for some reason as yet undetermined, the use of perfectly fresh preparations occasionally leads to atypical results. The medium contains $M/20$ phosphate, a concentration which lies between the $M/10$ solution used by Dixon & Elliott [1929] and which van Heyningen [1935] believes to be detrimental to animal tissues, and the $M/40$ solution which van Heyningen himself recommends. It was found necessary to use a fairly high concentration of buffer since the tissue itself has a distinctly acid reaction, and as much as 500 mg. of it had sometimes to be suspended in only a few ml. solution. The depression of the freezing point (0·31°), the pH (7·73 at 28°, electrometric), and the ratio Na^+/K^+ of this medium are the same as those of the haemolymph of normal active snails [see Baldwin & Needham, 1934].

The O_2 uptake of slices suspended in this solution fell off slowly with time, but the same thing happens in CO_2-$NaHCO_3$ Ringer [Baldwin & Needham, 1934], and seems to be due to fragmentation of the somewhat fragile tissue rather than to any deleterious effect of the suspension medium. Every care was taken to minimize fragmentation by keeping the shaking machine well lubricated and reducing the rate of shaking to about 70 oscillations per min. As a rule readings were taken only over the first $\frac{1}{2}$ hr., and very seldom over more than 1 hr., and during this period the respiration remained practically linear in nearly every experiment.

The "direct" technique described by Dixon [1934] was used for measuring the rate of respiration, the flasks being filled with pure O_2 and shaken in an electrically controlled bath maintained at 28°. This temperature has been repeatedly used for molluscan tissues and seems perfectly satisfactory [see for example Baldwin, 1935]. Where other methods have been employed they will be referred to in the text.

RESULTS

It was found that there is considerable variation in O_2 consumption from one hepatopancreas to the next, and this made it necessary that all the slices used in any given experiment should be prepared from one and the same animal. Since it is often impossible to cut uniform slices of the tissue, and as the organ is too small to allow of the preparation of a large number of slices from which the best could be selected, the measurements were usually made in duplicate. The results then showed a mean variation of the order of 10 %, and consequently each experiment was repeated several times and the results averaged. Each experiment mentioned in the protocols therefore refers to duplicate determinations on one and the same individual except in a very small proportion of cases. The rates of O_2 uptake are expressed throughout the paper in the usual Q_{O_2} notation, with reference to pH 7·7 and 28°. The quotients found for the control and experimental samples are represented by Q^r and Q^e respectively as a matter of convenience.

The effect of saccharides and other substances upon respiration

It has long been known that large quantities of glycogen can be stored in the hepatopancreas and that it disappears during starvation [von Fürth, 1903; Biedermann, 1911]. Probably much is used in muscular activity, for recent advances in muscle chemistry indicate that all muscles probably make use of closely similar mechanisms for their energy production. Very little is known about carbohydrate metabolism in *Helix*, although this form has been studied more than any other gastropod type. Schwartz [1935] and Wolf-Heidegger [1935] studied the effects of injecting adrenaline and insulin into snails, but could find no significant changes in the blood sugar level. Ermakov *et al.* [1935] on the other hand find that persistent hyperglycaemia develops after extirpation of the peripharyngeal ganglia or the injection of nicotine, while transient effects follow piqûre of the ganglia. These workers are of the opinion that the blood sugar is under nervous control.

In addition to glycogen a second polysaccharide, galactogen, is present in the snail. This substance, to which we must refer again, has been studied by May [1931–34], May & Kordowich [1932], and Schlubach & Loop [1937]. Investigations which began before the paper of Schlubach & Loop appeared are also in progress in this laboratory: the results lead us to believe that from the purely chemical point of view May's conclusions are not entirely reliable, and we hope to have definite evidence regarding this in the near future. In the most recent of his papers May has brought together the pertinent observations of earlier workers, and comes to the conclusion that galactogen is a polysaccharide consisting entirely of galactose units and having a peculiar importance in reproductive processes. There are indications in the work of Levene [1925] upon the slimy secretions of *H. aspersa* and *H. pomatia* that a polygalactose or perhaps an acetylated polygalactose may also be important in connexion with slime formation. May has shown that whereas glycogen is present in practically every part of the body of *Helix*, only the albumin gland contains galactogen, and here glycogen is absent. Galactogen accumulates in the albumin gland during the period prior to egg laying and is formed in quantities larger than can be accounted for by the amounts of galactose ingested, presumably being formed from some other sugar, such as glucose [May, 1934, 2]. The eggs themselves are remarkably rich in galactogen and contain no glycogen [May, 1932, 2], but if the animals are starved galactogen may disappear again and be utilized by the organism, but no eggs are then laid [May, 1934, 4]. It is likely therefore that glycogen (or glucose) and galactogen (or galactose) are interconvertible in the organism.

It should be mentioned in passing that one of the two samples of galactogen used in the present work was prepared from the albumin glands of a number of snails. The glands were crushed in alcohol and allowed to harden, boiled with 30 % KOH, precipitated with alcohol several times and finally purified by precipitation with alkaline $CuSO_4$ [May, 1934, 1], followed by repeated precipitation with alcohol. The other sample, kindly given to me by Dr N. W. Pirie, was obtained from the whole bodies in a similar way and freed from glycogen by treatment with salivary amylase. Both preparations were free from reducing sugars and contained over 90 % of galactogen.

The carbohydrates studied were added to the tissue in the form of a 2·5 % solution, the final concentration in the flask being *c.* 0·25 %. The results are summarized in Table I. It will be seen that most of the sugars added were without effect, since changes of less than 10 % cannot be regarded as significant on the basis of only a small number of experiments. Apart from the results

Table I. *Effect of carbohydrates on respiration of snail hepatopancreas*

| | Averages | | Increase |
Carbohydrate	Q^c	Q^e	%
Glycogen (5)	3·44	3·35	− 3
Galactogen (4)	3·51	· 4·68	+ 33
Inulin (3)	3·62	3·49	− 4
Raffinose (2)	2·54	2·63	+ 4
Maltose (2)	2·85	2·62	− 8
Lactose (2)	3·00	2·89	·· 4
Sucrose (2)	4·42	4·11	− 7
Fructosediphosphate (2)	3·48	3·31	− 5
Glucose (5)	2·93	3·01	+ 3
Mannose (4)	3·76	3·87	+ 3
Galactose (10)	2·66	3·85	+ 44
Fructose (4)	3·89	4·64	+ 19
Xylose (6)	2·93	3·42	+ 17
Arabinose (3)	3·00	2·94	− 2

Note: The figures in brackets refer to the number of experiments carried out with each substrate. *None of the experiments was carried out during the "winter period" (Dec.–Feb.)*; experiments performed at this time are considered separately.

obtained with galactose and galactogen, the general picture is not unlike that given by mammalian liver. Fructose and xylose cause small increases in the rate of respiration, but glucose, glycogen and fructosediphosphate are not oxidized. Correlated with this there is a very low rate of anaerobic glycolysis; a $Q_{M}^{N_2}$ value of about 1 was found at 28°, and Chapheau [1932] has found results of the same order (0·8–1·0) for the hepatopancreas of another mollusc, the oyster. These figures are comparable with those of 2–3 at 38° recorded for mammalian livers [see Krebs, 1933, 1]. The rates of respiration too are of the same order as for mammalian liver, for the Q_{O_2} values found in all the 92 control samples quoted in this paper (i.e. those to which no substrate was added) varied from −1·13 to −5·19 at 28°, with an average of −2·93, which is very like Chapheau's figure of *c.* −2 at 28° for the oyster hepatopancreas, and may be compared with the values of −3·4 to −18·7 for mammalian liver at 38° [Krebs, 1933, 1]. There is thus a general qualitative and quantitative resemblance between the activities of the molluscan hepatopancreas and the mammalian liver. Dorman's [1928] contention, that the invertebrate "hepatopancreas" and the vertebrate liver are homologous, could thus claim a certain amount of biochemical support.

But the parallel breaks down when we consider the large effects produced by galactose and galactogen which contrast interestingly with those of glucose and glycogen. It is possible that galactogen, which has a smaller effect than its parent sugar, may be converted into galactose before being oxidized. Neither galactosemonophosphate nor glucosemonophosphate has yet been tried, but it seems unlikely in view of the other results that the latter would be oxidized. Although the galactose ester has been shown not to be attacked by a yeast trained to act on galactose [Grant, 1935], it might be attacked here if phosphoric esters play any part in the suggested conversion of galactogen into galactose. The existence of an enzyme which catalyses the oxidation of galactose is very interesting since it seems that, in general, galactose is converted into glucose before being metabolized [see Grant, 1935]. The presence of such an enzyme in *Helix* must presumably be correlated with the fact that galactose is a major metabolite in this species.

Attempts to demonstrate the presence of dehydrogenase systems with the aid of the methylene blue technique were wholly unsuccessful. Whether the tissue was used in the form of a brei or as slices, the reduction time ran into many

hours in most cases, even in the presence of added substrates. It was therefore necessary to have recourse to manometric measurements again, and the effects of pyruvate, lactate, succinate and α-glycerophosphate have been studied. As Table II shows, only the last three of these substances produced considerable

Table II. *Effect of various substances on the rate of respiration of snail hepatopancreas*

Substrate ($M/100$)	Averages		Increase %
	Q^c	Q^e	
Pyruvate (3)	2·02	1·86	− 8
Lactate (3 + 5*)	2·51	3·04	+21
Succinate (3 + 4*)	2·80	4·00	+43
α-Glycerophosphate (7*)	2·33	3·32	+43
Alanine (3*)	3·26	4·16	+27

Note: The figures in brackets refer to the number of experiments carried out. Where the figure is marked with an asterisk the corresponding number of experiments was carried out during the "winter period".

increases in the rate of respiration. That pyruvic acid is without effect indicates that it is not converted by the tissue into succinic acid at any considerable rate, though this transformation is known to take place in moulds [Butkewitsch & Fedoroff, 1929] and in yeast [Wieland & Sonderhoff, 1932]. The transformation also occurs in mammalian kidney, but not in liver [Elliott, 1937].

In one experiment in which the Thunberg technique was used, 500 mg. fresh slices were set up with buffer, $M/100$ succinate and 2 ml. 1/20,000 methylene blue at 28°, and no less than 80 min. were required for decoloration of the dye. Nevertheless, as we have seen, the rate of O_2 uptake of the slices is of the same order as for slices of mammalian liver. From the data of the experiment just quoted a Q value of about −0·03 can be calculated to correspond to the rate of reduction of the methylene blue, whereas the Q of normal respiration is about −3. O_2 is therefore reduced about 100 times as fast as methylene blue. In the case of yeast [Wieland & Claren, 1932] there is a factor of about 10, and the slowness of methylene blue reduction is due to the failure of the dye to penetrate into the cells. Methylene blue, indeed, is used as a diagnostic reagent for determining the viability of yeast cells. If these cells are disintegrated and their contents thereby made accessible to the dye, a rapid reduction begins at once. Similarly, mammalian liver reduces methylene blue more rapidly in brei than in slice form, but in the case of the snail tissue no more rapid reduction was obtained by using brei preparations.

It seemed worth while to test the hepatopancreas for the presence of a possible inhibitory substance, and this was done by comparing the rates of reduction of methylene blue by washed, chopped frog muscle in the presence of succinate, with and without the addition of aqueous extracts of hepatopancreas, but the reduction times were identical within the limits of experimental error. Although methylene blue is known to inhibit most dehydrogenases to a greater or less extent [Meldrum, 1934, p. 39], it seems unlikely that inactivation of this kind could account for the large difference found here.

The addition of methylene blue at concentrations up to 1/5000 had but little effect on the O_2 uptake of surviving hepatopancreas slices, and cresyl blue was not much more active in this respect (Table III). The mean increase found was of the order of only 20 %, which is very different from the admittedly exceptional increases of 20–30 times found by Barron [1929] for the respiration of red blood cells. Now the addition of an auxiliary O_2 carrier such as methylene blue can

only bring about an increase in the rate of O_2 absorption of a tissue if the rate of action of the reducing systems of that tissue is limited by that of the normal O_2-carrying systems, suggesting that in the present case the rate of O_2 transport in the tissue is not the limiting factor. This possibility was investigated by reducing the rate of respiration to about one-fifth by poisoning with $5.10^{-3}M$ cyanide. The experiments showed conclusively that even then the respiration is not stimulated to any significant extent ($+8\%$) by the addition of 1/5000 methylene blue. Dixon & Keilin [1936] have pointed out, however, that the inhibition produced by cyanide is not reversible under all conditions, but it is very improbable that irreversible inhibition can have played a significant part here since, in experiments in which no precautions were taken to prevent the distillation of HCN from the suspension medium into the alkali used for CO_2 absorption, the inhibition fell off very markedly in the course of time.

Table III. *Effects of dyestuffs on respiration of hepatopancreas*

		Averages		Increase
Dye and concentration	Substrate	Q^c	Q^e	%
1/50,000 methylene blue	Galactose (3)	2·88	2·95	+ 2
1/5000 methylene blue	Succinate (3)	3·60	4·22	+17
1/5000 cresyl blue	Succinate (3)	3·04	3·93	+29

(Concentration of substrate $M/100$ in all cases.)

The failure of methylene blue to exert its usual effect when added to hepatopancreas tissue need not, however, be taken as conclusive evidence that the mechanisms of cellular respiration here are essentially different from those found in other tissues. It must be supposed that methylene blue fails to reach the reducing systems for some reason, though not, apparently, because it fails to penetrate into the cells. As we shall see, there is reason to think that the oxidation of succinic acid, at any rate, is catalysed by a typical succinic dehydrogenase in this as it is in other tissues.

A complete survey of the individual dehydrogenases has not been attempted. The data of Table II suggest that powerful succinic and α-glycerophosphate dehydrogenases are present, together with a rather weaker lactic enzyme, while those of Table I may indicate the presence of a powerful dehydrogenase for galactose. In view of the recent work of Szent-Györgyi [1937] and Krebs & Johnstone [1937], for example, it seemed desirable to carry the study of the supposed succinic dehydrogenase a little further. This was done by studying the influence upon the respiration of surviving hepatopancreas slices in the presence of succinate of (*a*) narcotics, which inhibit the majority of dehydrogenases, and of (*b*) malonate. which inhibits the succinic enzyme competitively and rather specifically.

The concentrations of various narcotics required to reduce to 50 % the O_2 uptake of washed frog muscle in the presence of succinate have been determined by Sen [1931], and in the present case four different narcotics were used at the concentrations given by this author. Well washed slices of the tissue were suspended in the usual phosphate solution in the presence of $M/100$ succinate, with and without the addition of phenylurea, symm. diethylurea, urethane and phenylurethane. The data in Table IV (*a*) show that, although different individual narcotics produce varying degrees of inhibition, the average of all the results is very close to the expected 50 % inhibition, and it therefore seems reasonably likely that the hepatopancreas oxidizes added succinate by means of a typical succinic dehydrogenase. More conclusive results were obtained

Table IV. *Effects of certain respiratory inhibitors*

	Inhibitor	Molarity	Averages Q°	Q^s	Increase %
(a)	Phenylurea (2)	0·028	4·71	2·78	− 41
	Diethylurea (2)	0·350	3·67	1·79	·· 51
	Urethane (2)	0·670	3·67	1·02	− 72
	Phenylurethane (2)	0·003	3·53	2·79	− 21
(b)	Malonate (1)	0·010	4·31	4·08	− 5
	,,	0·020	4·41	3·54	− 20
	,,	0·050	4·49	3·11	− 31
	,,	0·100	4·52	2·40	− 47
(c)	Malonate (3)*	0·100	2·09	1·61	− 23

(a) group: Av. 46%

Substrate $M/100$ succinate in all cases except that marked with an asterisk, in which no substrate was added.

in experiments in which malonate was used. Of the respiration taking place in the presence of succinate, nearly 50 % is due to the addition of that substance (Table II), and about the same percentage is inhibited by $M/10$ malonate (Table IV (b)). Quastel & Wooldridge [1928] found that the addition of $M/14$ malonate inhibits more than 95 % of the activity of the succinic dehydrogenase of *Bact. coli* in the presence of $M/140$ succinate. The fact that the relative concentrations of substrate and inhibitor are the same in both these cases very strongly suggests that the respective dehydrogenases must also be similar. The effects of $M/10$ malonate upon the basal respiration of hepatopancreas slices was also tested (Table IV (c)) and it was found that, even in the absence of added succinate, 20–25 % of the respiration is inhibited, suggesting that the succinic dehydrogenase system may be in some way concerned in a considerable proportion of the basal respiration of this tissue.

Metabolism during the winter period

It was found in the course of experiments carried out between December 1936 and February 1937 that the usual effect of galactose upon the rate of respiration cannot in general be observed at this time of year. The more or less complete disappearance of this effect during what might be called the "winter period" is interesting from several points of view. Table V (a) gives a series of confirmatory data obtained during the winter period of 1937–38, and it will be observed that although a fairly, though not typically, large increase occasionally follows the addition of galactose to the tissue slices (Exps. 146, 148), the effect is absent in most cases. These data may be compared with those obtained at other times of year and shown in Table IV (b). The average increase for the whole series shown in Table V (a) is only 10 %, a value which is just on the borderline of significance. There is no reason to suppose that the winter period is associated with any peculiarities of metabolism so far as the other systems which were studied are concerned, for all these were found still to be active (Table VI). This suggests that the suppression of the galactose effect is not attributable to a breakdown on the part of the O_2-carrying system (i.e. the cytochrome + cytochrome oxidase system or its counterpart). The breakdown of the mechanisms by which galactose is ordinarily oxidized might be due (a) to the absence of some special carrier or coenzyme, or (b) to the absence of the enzyme itself. The first of these possibilities has not been tested since it was not found possible to extract any of the enzymes from the tissue itself.

Table V. *Effect of galactose on respiration of snail hepatopancreas*

Exp. no.	Q^c	Q^s	Increase %
		(a) During winter	
143	4·24	4·20	− 1
144	2·42	2·50	3
145	1·76	1·85	5
146	1·97	2·78	41
147	1·88	2·06	10
148	1·13	1·35	19
149	2·32	2·52	9
150	2·23	2·54	14
151	1·58	1·70	8
Averages	2·17	2·39	10
		(b) At other periods	
8	3·85	5·31	38
15	2·51	2·62	4
19	2·40	3·52	47
22	2·18	4·06	86
30	3·38	5·92	75
64	2·22	3·10	40
65	3·22	4·79	49
66	2·45	2·91	19
152	2·45	3·33	36
153	1·95	2·93	50
Averages	2·66	3·85	44

Table VI. *Metabolism during winter period*

Substrate	Mean increases %		Data in table no.
	"Summer"	"Winter"	
Galactose	44	10	V (a, b)
Alanine	—	27	II
Succinate	52	38	II
Lactate	19	23	II
α-Glycerophosphate	—	43	II

The alternative view involves consideration of the galactose enzyme as an "adaptive" one, disappearing during the winter for some reason as yet unknown, and reappearing in the spring, perhaps in response to the presence of galactose in the food which is then ingested. There is now a fair body of evidence to show that bacteria can develop enzymes of this kind in response to the presence of certain particular substances in the culture media, and even among Metazoa there are examples of the production of adaptive enzymes [Stephenson, 1937; Yudkin, 1938].

It seems possible that the suppression of the oxidation of galactose by the hepatopancreas during the winter period may be a physiological, seasonal change, correlated with the fact that the snail normally spends this part of the year in hibernation. It is not known whether the seasonal changes in metabolism which take place in certain animals are associated with corresponding "adaptive" changes in enzymic activity, even in the extreme cases in which hibernation takes place. Indeed, we know very little about the metabolism of hibernation [Gorer, 1930]. It is generally believed that in the cases of the hedgehog and the marmot, the metabolism of carbohydrate is almost entirely suppressed in favour of that of fat during the winter sleep; but whether there are any changes in the enzymes concerned we cannot say. Considerable metabolic differences exist between winter and summer frogs [see Tigerstedt, 1914, and Adler, 1921, for example], and seasonal variations are known even in animals which do not pass

the winter in a state of inanition. Sherwood [1936], for instance, has found small but perfectly definite seasonal changes in the basal metabolic rate of rats. Seasonal variations such as these may well be associated with changes in enzymic activity, and the disappearance, or at any rate the suppression, of the oxidation of galactose in "winter" snails may perhaps be an example of this.

There is reason to believe, however, that galactose can be utilized in some way by winter snails, for galactogen may disappear from the albumin glands of starved specimens after the stores of glycogen have been depleted [May, 1934, 4]. If, as seems to be the case, we must regard galactogen as a substance which is stored up in preparation for the egg-laying season, suppression of the oxidation of galactose during hibernation might be regarded as a device for conserving this polysaccharide as far as possible.

Oxidase systems

The presence of xanthine oxidase in the hepatopancreas of *Helix* has already been demonstrated by Przyłecki [1926] and confirmed by Baldwin & Needham [1934], but it could not be shown by the technique used in the present experiments. No increase in the rate of respiration (-2%) could be found following the addition of $M/100$ hypoxanthine to tissue slices, or to a preparation obtained by grinding the fresh tissue with half its own weight of sand and five volumes of phosphate buffer, centrifuging, half-saturating the centrifugate with ammonium sulphate, centrifuging again and finally taking up the precipitate in phosphate buffer. It is known, however, that xanthine oxidase has a great affinity for its substrate. For the xanthine oxidase of milk, Dixon & Thurlow [1924] give a Michaelis constant of $3.10^{-5}M$, and it is quite possible that in the present experiments the enzyme was already saturated with its substrates, considerable amounts of which are believed to be present in the hepatopancreas tissue [Wolf, 1933].

The presence of amino-acid deaminase was readily demonstrated. Since pyruvate had already been found to have no effect upon the rate of respiration, alanine was a suitable substrate for these experiments, and a $M/100$ solution of the *dl*-acid was employed. The results are shown in Table II. The presence of the deaminase was also detected by shaking 5.23 g. washed slices with 500 mg. alanine in 25 ml. CO_2-$NaHCO_3$ Ringer [see Baldwin & Needham, 1934] to which 1 ml. saturated As_2O_3 had been added [cf. Krebs, 1933, 2]. The shaking was continued for 5 hr. at $28°$ and the fluid then deproteinized with 5 ml. 30% trichloroacetic acid. When cold, the filtrate received 10 ml. saturated 2:4-dinitrophenylhydrazine in $2N$ HCl, and crystallization began at once. The whole was left in the refrigerator till next day and the crystals were then collected on the centrifuge, washed twice with $N/4$ HCl and three times with distilled water and finally dried *in vacuo*: M.P. $208°$. After recrystallization from ethyl acetate the purified product came out in hexagonal plates, M.P. $216°$. Found: C, 40.4; H, 3.1; N, 21.0%. Calc. for the 2:4-dinitrophenylhydrazone of pyruvic acid: C, 40.3; H, 2.99; N, 20.9%. An entirely similar product was obtained by allowing an acetone powder preparation of the hepatopancreas to act upon alanine [cf. Krebs, 1935].

Colour tests were carried out on *p*-phenylenediamine, *o*-phenylenediamine, catechol, 3:4-dihydroxyphenylalanine and tyrosine. When well washed slices of the hepatopancreas were added to dilute aqueous solutions of these substances a deep purple coloration was produced within $\frac{1}{4}$ hr. in the case of *p*-phenylenediamine, but the other reagents showed no appreciable colour change after 24 hr.

If the slices were not thoroughly washed, weak positive reactions were given also by catechol and 3:4-dihydroxyphenylalanine, and Pugh [1930] has found that these substances, but not monohydric phenols, are also oxidized by extracts prepared from the large black slug, *Arion ater*. In both these cases tyrosinase must be regarded as absent. But it seems difficult to attribute the powerful and decidedly specific oxidation of p-phenylenediamine to any enzyme other than the cytochrome (indophenol) oxidase.

Confirmation of the presence of this enzyme was obtained manometrically by measuring the increase in O_2 uptake resulting from the addition of a few mg. solid p-phenylenediamine to slices of hepatopancreas respiring in $M/100$ succinate. Characteristically large increases (average $+360\%$) followed the addition of this reagent. The effect was counteracted to the extent of 70–80 % by the simultaneous addition of $10^{-2} M$ cyanide, and the presence of cytochrome oxidase thus seemed to be very probable. Further confirmation of this was obtained by taking advantage of the fact that cytochrome oxidase is powerfully inhibited by cyanide. In the majority of animal tissues it seems that 60–80 % of the total respiration is inhibited by $10^{-3} M$ cyanide and therefore mainly due to the cytochrome system.

The results of much early work on cyanide inhibition were invalidated because no precautions were taken to prevent distillation of cyanide from the suspension medium into the alkali used for CO_2 absorption, but Krebs [1935] has described equilibrating solutions which overcome this difficulty. These were used in the present experiments, and with very satisfactory results, for whereas the inhibition passed off with time if this precaution was not taken, no such effect was detectable when Krebs's solutions were employed. The results of the experiments, which were carried out in the presence of $M/100$ succinate, are shown in Fig. 1. They closely resemble those obtainable with many other animal
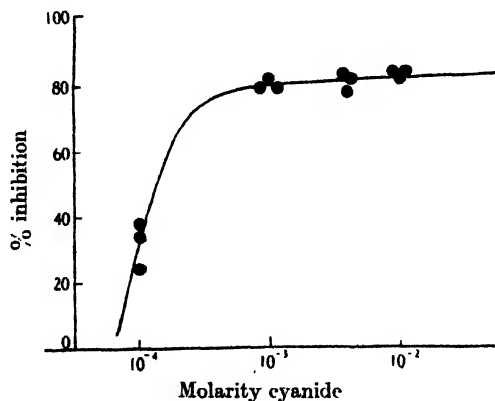
Fig. 1. Effect of cyanide on respiration of hepatopancreas.

tissues. A maximal inhibition of 80–85 % is reached at a cyanide concentration of $10^{-3} M$, and the concentration of cyanide required to inhibit 50 % of the total cyanide-sensitive respiration is about the same as that for cytochrome oxidase. It therefore seems reasonably certain that cytochrome oxidase is present in the hepatopancreas.

Perhaps there has been no more fundamental contribution to the subject of cellular respiration than the discovery by Keilin [1925] of cytochrome and of the part which it plays in living tissues [Keilin, 1929; 1933]. It seems, indeed, that

the cytochrome + oxidase system is of almost universal occurrence in aerobic organisms, but its functional importance has been definitely established in only a few particular tissues. Although various unicellular organisms and some mammalian tissues have now been examined it must be owned that the lower Metazoa have scarcely been studied at all from this point of view, although Keilin [1925] has studied the biological oxidation and reduction of cytochrome in the wing muscles of the wax-moth, *Galleria*.

An attempt was made to demonstrate the presence of cytochrome by micro-spectroscopic examination of slices of the hepatopancreas. Whereas Keilin's [1925] finding of cytochrome in the muscles of the radula was readily confirmed, no trace of cytochrome could be found in the hepatopancreas. In this respect again the hepatopancreas resembles mammalian liver. The tissue is so darkly coloured that only small thicknesses of it could be examined, and it is therefore not impossible that minute amounts of cytochrome may have been present but evaded detection. The only visible band corresponded to the α-band of reduced helicorubin, and this was visible even after the tissue, which was used in the form of thin slices piled on top of each other, had been subjected to very vigorous washing.

This pigment, helicorubin, is very widely distributed. It occurs in the gut juice of starving specimens of *Helix*, and according to MacMunn [1885], who described it under the name of enterohaematin, it is present also in the gut contents of many other gastropods and also in some crustaceans. Pantin [1932] states that it is to be found also in the gut of an annelid worm, *Aphrodite*. It appears to be made in the hepatopancreas and poured out into the gut, and "the helicorubin in the liver of the crayfish appears to be exactly the same as mol-luscan helicorubin" [Anson & Mirsky, 1925]. In *Helix* at any rate it must probably be of physiological importance, for if a starving snail be fed on filter paper, no trace of helicorubin can be detected in the faecal mass. This suggests that the pigment is not to be regarded simply as a waste product, analogous perhaps to the bile pigments of the mammals, but that it is actively retained in the body. In view of the fact that it is autoxidizable at the rather acid pH of the gut contents, Anson & Mirsky [1925] suggested that it might act as a respiratory pigment, but since it is found in the gut and the hepatopancreas it is a little difficult to see how it could play any important part in the respiration of the animal as a whole.

It seems not inappropriate at this point to put forward the tentative hypo-thesis that, while cytochrome is in all probability the chief ultimate H acceptor in many tissues, it is replaced by helicorubin in the hepatopancreas of *Helix*, and perhaps elsewhere. Experiments are in progress with a view to testing the possible truth of this hypothesis, and in the meantime there is some apposite information. It is already known that cytochrome is replaced by other haematin compounds in certain tissues, among which mammalian liver is included. Keilin [1929] has shown that its place is taken by another type of haemochromogen in facultative anaerobes, the liver cells of mammals and in the cells of some gastropods. The actiniohaematin of the Actinia is also a pigment of this kind [Roche, 1936, 2; see also the review by Roche, 1936, 1]. Helicorubin itself is a haematin pigment, the haem of which is identical with that of haemoglobin [Dhéré & Vegezzi, 1917; Anson & Mirsky, 1925] and very like that of cyto-chrome *c*, if not identical with it [Hill & Keilin, 1930]. In slightly acid solutions it is autoxidizable, a property which it shares only with haemoglobin, and with cytochrome in rather strongly alkaline solutions. Near the neutral point it loses this property but is then oxidizable in the presence of cytochrome oxidase,

just as is cytochrome itself under physiological conditions [Keilin, 1925, 1933]. These properties fit it, more than any other known haematin compound, to take the place of cytochrome.

Evidence for the presence of cytochrome oxidase in the hepatopancreas of *Helix* has already been presented and there can be little doubt therefore that helicorubin, which is certainly present in this tissue, can be oxidized there under the conditions obtaining *in vivo*. With regard to its possible reduction under biological conditions, some preliminary experiments have already shown that it is very rapidly reduced by traces of cysteine, and rather more slowly by certain biological systems. Further work in this direction is in progress.

SUMMARY

1. The results of a number of experiments on the respiratory metabolism of the hepatopancreas of *Helix pomatia* show that on the whole this tissue resembles the liver tissue of vertebrates fairly closely, but differs markedly from it in that its rate of respiration is much increased by galactose and galactogen.

2. Succinate, lactate, α-glycerophosphate and alanine also increase the rate of respiration.

3. During the winter the oxidation of galactose is largely suppressed, though succinate, lactate, α-glycerophosphate and alanine still produce their usual effects. It is suggested that this may be physiological seasonal change.

4. The oxidation of succinate appears to be due to a typical succinic dehydrogenase. This is inhibited by narcotics and by malonate in the same way and to the same extent as the succinic dehydrogenase of other tissues.

5. No rapid reduction of methylene blue by the tissue could be demonstrated under any of the conditions tried, nor could the respiration of normal or cyanide-poisoned tissue be accelerated by means of methylene blue.

6. Cyanide in $M/1000$ solution inhibits 80 % of the respiration of the hepatopancreas, and the inhibition seems to be attributable to the poisoning of cytochrome oxidase. The presence of this enzyme seems to be highly probable.

7. No cytochrome could be detected spectroscopically in the tissue; the only haematin compound detectable in this way is helicorubin. A possible function of this pigment is tentatively suggested and discussed.

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CLXV. COMPOSITION OF THE MILK FROM THE BREASTS OF NEWLY-BORN INFANTS

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It is well known that most infants of both sexes secrete milk when newly born, the secretion being termed witches' or sorcerers' milk. Halban [1904] described the phenomenon as a puerpural involution in the mammary glands of the newly born infants. Billard [1837] considered the secretion to be imperfect milk loaded with leucocytes, often ending in abscesses. Schlossberger [1852] has given an analysis of one sample, viz. total solids 3·25, fat 0·82, protein and sugar 2·83 and ash 0·5 %. Three analyses by Gessner [1867] show the secretion to contain total solids 3·7–10·6, fat 0·8–1·5, total protein 1·1–2·8 and lactose plus ash 1·9–6·4 %.

In the present investigation, 5 samples of such secretions were collected from infants of both sexes between 9 and 17 days old and submitted to as comprehensive an analysis as the small yields obtained permitted. The milk was obtained by gentle squeezing of the enlarged breasts, the milk then being sucked up by a pipette and transferred to small glass bottles containing a trace of formalin. The yields were variable but in no case exceeded 1·8 g.

By suitable management of fractions and adoption of microchemical technique, the following analytical data were collected: total solids, nitrogen distri-

Table I. *Composition of milk from breasts of infants*

Sample No.	1	2	3	4	5
Sex	M. and F.	M.	F.	M.	F.
Age (days)	8 (M.) and 17 (F.)	9	14	10	9
Yield (g.)	1·20	0·35	0·84	1·77	0·65
As % of milk:					
Total solids	8·67	12·50	7·36	9·76	10·57
Protein (N × 6·38)	3·40	7·23	4·60	2·68	8·31
Lactose	1·80	1·56	1·56	2·67	1·00
(as glucose)	0·99	0·86	0·86	1·47	0·55
Fat + ash, etc. (by difference)	3·47	3·71	1·20	4·41	1·26
Cl	0·14	0·10	0·14	0·10	0·12
Total N	0·53	1·13	0·72	0·42	1·30
Protein N	0·34	0·62	0·51	0·28	0·71
Casein N	0·22	0·52	0·36	0·21	0·56
Non-protein N	0·19	0·51	0·21	0·14	0·59
Osmotic equivalents:					
Lactose equivalent of Cl	2·74	1·96	2·74	1·96	2·35
Lactose equivalent of N.P.N. (calc.)	2·50	4·30	2·70	2·40	3·60
As % of total N:					
Protein N	65	55	70	67	55
Casein N	40	46	49	33	43
Non-protein N	35	45	30	34	45
Casein in protein %	63	84	70	50	79
Peroxidase	+	+	+	+	+
Phosphatase (units per 0·5 ml.)	7·6	7·4	7·5	5·1	6·9

bution, sugar and chloride content and evidence of the presence of peroxidase and phosphatase. The weights of samples were unfortunately too small to determine fat and mineral distribution; the amount of ash was in all cases too small to be determined accurately.

Total solids were determined by drying at $100°$. Nitrogen distributions were studied by separating the casein at pH 4·6 with an acetate buffer and centrifuging, and total protein was determined by precipitation with 4 % trichloroacetic acid at $70°$; all the nitrogen fractions were determined by micromethods. Sugars were determined by Maclean's method for blood sugar. Peroxidase was tested for by the benzidine-H_2O_2 method and phosphatase by the Kay & Graham [1935] technique. Table I gives the results obtained for the 5 samples.

All samples appeared to contain fat since they showed the property of creaming on standing; no churning of the fat had occurred during transit in the post. The fat content was not determined.

Discussion of results

The total solids content was regularly higher than that reported by other workers, except a value for one sample given by Gessner. The highest total solids occurred around the 9th day, protein accounting for most (60-80 %) of the solids. It is probable that the secretion is at first watery, rises in total solids and protein content to the 9th day, and then decreases in solids slowly and in protein rapidly, due to resorption in the following days.

The resorption period yields a secretion which is higher in lactose and chloride than the pre-resorption period. If the secretion obeys the same osmotic laws as normal milk, it can be stated that the higher non-protein nitrogen content of the secretions containing high total nitrogen contents balances osmotically the low contents of sugar and chloride. This was roughly tested out in the following manner. In normal milk with only 6 % of the total nitrogen as non-protein nitrogen, the relationship between lactose and chloride is expressed by the formula:

$$\text{Lactose } \% + 19·6 \text{ Cl } \% \text{ (A.)} = 7·0 \text{ [Davies, 1932]}.$$

None of the present secretions obey this relationship, owing to the high non-protein nitrogen content. The value 7·0-A. was plotted against the percentage of non-protein nitrogen for each case. This gave points which lay roughly on a straight line represented by the equation:

$$\text{Lactose equivalent of non-protein nitrogen} = \text{N.P.N. } \% \times 3 + 2.$$

Sugar, chloride and non-protein nitrogen are thus responsible for most of the osmotic pressure of these secretions.

Protein. The amount of the total true protein represented by casein was variable, ranging from 50 to 84 %. Generally, casein percentage was highest when total nitrogen was highest. The non-casein protein, according to qualitative precipitation tests, contained globulin, as saturation of casein-free filtrates with Na_2SO_4 and $MgSO_4$ gave flocculent precipitates. The amount of non-casein protein was lowest for samples with high non-protein nitrogen content.

General. The secretions bear some resemblance in composition to those from the udders of pregnant heifers and dry non-pregnant cows [Woodman & Hammond, 1923], especially the latter. Close similarity exists in the low lactose, high total protein with low proportion of casein to total protein, and high non-protein nitrogen content. The appearance of globulin in amounts above those found in normal milk in the above and in infant secretions is undoubtedly associated with the abnormal functioning of the mammary glands, such as is found

before and immediately after parturition (colostrum). The secretion in infant breasts tends to resemble colostrum in composition, the main points of difference being the very low lactose and high non-protein nitrogen which does not occur in the latter.

The secretions gave strong peroxidase reactions, due probably to the presence of leucocytes which were observed in all samples by microscopic examination. The phosphatase content was of the same level as that found in normal cow's milk.

SUMMARY

The analyses of 5 samples of secretions from the breasts of newly-born infants from 8 to 14 days old are reported. The total solids, which range from 7·4 to 12·5 %, consist mostly of protein and lactose. Compared with normal milk, the secretions are low in lactose but high in globulin and non-protein nitrogen. The secretions contain appreciable amounts of peroxidase and phosphatase.

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CLXVI. THE SPECIFICITY OF ANEURIN AND NICOTINAMIDE IN THE GROWTH OF *STAPH. AUREUS*

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It has been shown [Knight, 1937, 1, 2, 3] that nicotinamide and aneurin (or certain closely related derivatives corresponding to these two compounds) are necessary for the growth of typical strains of *Staph. aureus* in a medium of known chemical composition.

The activities of several compounds closely related to nicotinamide and aneurin which were previously tested [Knight, 1937, 3] showed that only a limited departure from these two chemical structures was compatible with the maintenance of growth activity. It was further found that a mixture of a pyrimidine and a thiazole, both having substituents corresponding to those of the component pyrimidine and thiazole rings of aneurin, could be used in place of aneurin itself. The behaviour of the further series of compounds recorded in the present paper emphasizes the specificity of both the nicotinamide and the aneurin structures in the growth requirements of *Staph. aureus*.

Technique

The methods used for the growth tests were the same as those previously described [Fildes *et al.* 1936; Fildes & Richardson, 1937]. The test organism was a typical strain of *Staph. aureus* which could not grow on the basal amino-acid medium without the further addition of nicotinamide and aneurin (or the equivalent pyrimidine + thiazole; see below).

The substances to be tested fell into four categories:

(1) Aneurin analogues: as substitutes for the complete aneurin molecule.

(2) Pyrimidine series: as substitutes for the pyrimidine component of aneurin.

(3) Thiazole series: as substitutes for the thiazole component of aneurin.

(4) Pyridine series: as substitutes for nicotinamide.

The compound to be tested was added in serial dilution to the basal medium which was nutritionally adequate in all respects except for that component, the substitute for which was under test. Thus of the complete group of nutrients required for growth only one component was varied at a time. Pyridine derivatives (as substitutes for nicotinamide) were always tested in presence of an excess of aneurin ($1 \times 10^{-7} M$). Similarly aneurin analogues or derivatives (pyrimidines and thiazoles) were tested in the presence of an excess of nicotinamide ($1 \times 10^{-6} M$). When the component parts of the aneurin molecule were being investigated separately, i.e. either the pyrimidine or the thiazole moiety

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was being varied, then an excess of the corresponding thiazole or pyrimidine was added. Thus when testing thiazoles, 2-methyl-4-amino-5-aminomethyl-pyrimidine was used as the complementary active pyrimidine; conversely when testing pyrimidines 4-methyl-5-β-hydroxyethylthiazole was used.

Growth was aerobic and incubation was continued for 3 days to allow time for slow utilization, if it occurred, to become apparent. In general the picture of activity shown within 24 hr. was not altered, qualitatively or quantitatively, on further incubation. Only in two cases have compounds apparently inactive at 24 hr. been found to permit growth on longer incubation: these compounds were methyl nicotinate [Knight, 1937, 3 and Table IV] and 4-methyl-5-vinyl-thiazole (see No. 14, Table III).

The results obtained are collected in Tables I–IV; for completeness and convenience of comparison the chief results previously obtained are also included. Fig. 1 shows the structural formulae of aneurin and its components for convenience of reference.

4-Amino-5-aminomethyl-2-methylpyrimidine

4-Methyl-5-β-hydroxyethylthiazole

pyrimidine ring

thiazole ring

Aneurin chloride
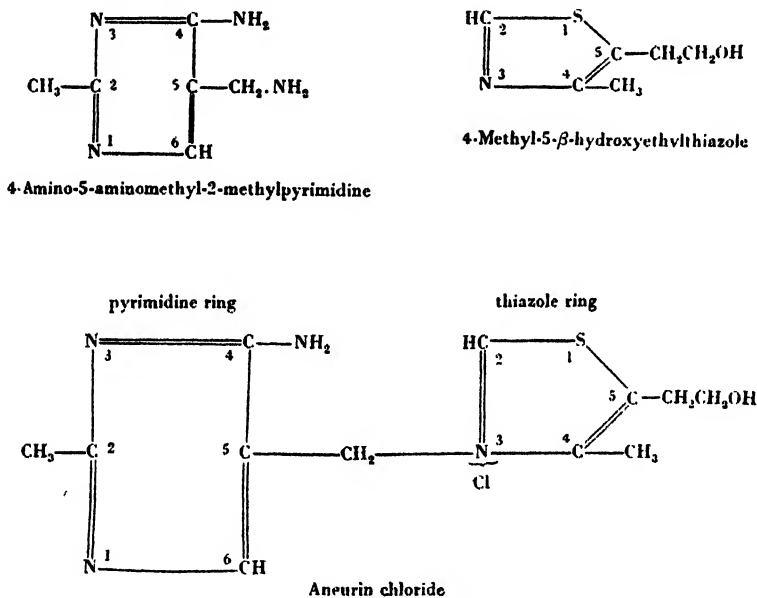
Fig. 1.

Aneurin analogues

In all this work where the typical active substances (e.g. aneurin) are of high potency it is necessary when testing closely related substances to be certain that apparent activities are not due to contamination by active impurities. In these circumstances failure to obtain growth is acceptable evidence of lack of activity, whereas an apparently lowered potency must be regarded sceptically, until it can be shown, either by repeated purification leading to no alteration of activity, or by the method of synthesis, that an active impurity is not the cause of the observed growth.

The much lowered potency of the "*isoaneurin*", about 1/10,000th of the activity of aneurin, is probably real. From the method of preparation [Andersag & Westphal, 1937, pp. 2050–1] no contamination with aneurin itself seems

Table I. *Aneurin analogues*

	Conc. M	Growth 24 hr.
Aneurin chloride HCl	1×10^{-8}	+ + + +
,,	2×10^{-9}	+ + +
,,	4×10^{-10}	+ +
,,	8×10^{-11}	?
*iso*Aneurin	1×10^{-5}	+ + +
,,	1×10^{-6}	?
,, + correct pyrimidine $10^{-7} M$	1×10^{-7}	+ + +
,, + correct pyrimidine $10^{-8} M$	1×10^{-8}	?
Chloroaneurin	1×10^{-5}	+ + +
,,	1×10^{-6}	?
,,	1×10^{-7}	0
,, + correct pyrimidine $10^{-7} M$	1×10^{-8}	+ + + +
,, + correct pyrimidine $10^{-7} M$	1×10^{-9}	+ + +
Aneurin minus hydroxyethyl group	1×10^{-5}	0
,, ,, ,, + correct thiazole $2 \times 10^{-7} M$	1×10^{-6}	+ + + +
2-Methylaneurin	1×10^{-5}	0
,, + correct thiazole $1 \cdot 0 \times 10^{-7} M$	1×10^{-6}	+ + +

Aneurin: synthetic specimen (Merck).

*iso*Aneurin: having CH_3 at 6 instead of 2 in the pyrimidine. (I.G. Farbenindustrie; transmitted by Dr A. Lwoff.)

Chloroaneurin: having Cl instead of CH_3 at 2 and CH_3 at 6 in the pyrimidine. (Drs Bergel & Todd.)

Aneurin minus hydroxyethyl group: lacking the 5-β-hydroxyethyl group of the thiazole ring. (Bergel & Todd.)

2-Methylaneurin: having an additional CH_3 at 2 in the thiazole. (Bergel & Todd.)

In this and in subsequent tables:

+ + + +, + +, + +, etc.: = number of + signs proportional to related mass of growth.

tr. = trace of growth just visible to the naked eye.

? = just detectable effect visible to naked eye.

0 = no detectable effect at concentration shown.

possible. Using the "*iso*aneurin" as a source of the thiazole only, that is, adding also 2-methyl-4-amino-5-aminomethylpyrimidine, there was an enhancement of activity, although not reaching the level expected from equivalent active pyrimidine + thiazole mixtures.

Similarly the much reduced potency of the "chloroaneurin" is probably real and not due to active impurity. Bergel & Todd [1937] reported that this compound apparently possessed some vitamin activity on one occasion, but further examination of the material failed to confirm this. The chloro compound was a potent source of the thiazole for *Staph. aureus* when tested in the presence of the correct pyrimidine.

The other two aneurin analogues tested have both altered substituents in the thiazole ring and their lack of activity is confirmed by the inactivity of the corresponding thiazoles in thiazole-pyrimidine pairs (Nos. 6 and 12, Table III). These two analogues were found by Bergel & Todd [1937] to be devoid of vitamin activity for rats.

Pyrimidines

The results with the new pyrimidines tested confirm the conclusions of the previous work [Knight, 1937, 3]. The apparent activity of 2-methyl-4-methoxy-5-aminoethylpyrimidine (No. 4, Table II) was undoubtedly due to contamination with the corresponding active 4-amino compound (No. 1, Table II). This is clear from the mode of preparation of the 4-methoxy compound [Andersag & Westphal, 1937, pp. 2046–7] which involves the separation of a 4-methoxy compound from the corresponding 4-amino compound by different solubilities

in methanol. This might easily permit sufficient contamination of the former by the latter to account for the apparent activity; contamination of less than 0·1 % would suffice.

The observed effects with all the pyrimidines tested show that the essential substituents of the ring for activity are: a CH_3 group at position 2; an NH_2 group at 4; and at position 5 a CH_3 group substituted in certain ways. Thus at 5, —CH_2NH_2, —CH_2OH and —$CH_2NH.CSH$ permit growth; but —$CH_2CO.NH_2$ does not, nor does an unsubstituted —CH_3.

These results have a bearing on the question of whether *Staph. aureus* requires the complete molecule for its growth activity and whether it does in fact join an active pyrimidine and thiazole together to make aneurin when given the two components. The —CH_2— at position 5 is that which forms the link between the pyrimidine and thiazole rings in aneurin. Since the results in Table II show that the nature of the groups attached to this 5-CH_2— group

Table II. *Pyrimidines*

	Conc. M	Growth 24 hr.
1. 2-Methyl-4-amino-5-aminomethylpyrimidine	1×10^{-8}	+ + + +
	2×10^{-9}	+ +
	4×10^{-10}	+
	8×10^{-11}	?
2. 2-Methyl-4-amino-5-hydroxymethylpyrimidine	1×10^{-8}	+ + + +
	1×10^{-9}	+ +
	1×10^{-10}	±
3. 2-Methyl-4-amino-5-thioformamidomethylpyrimidine	1×10^{-6}	+ + + +
	1×10^{-7}	+
	1×10^{-8}	0
4. 2-Methyl-4-methoxy-5-aminomethylpyrimidine	1×10^{-7}	+ +*
	2×10^{-8}	+
	4×10^{-9}	0
5. (2-Methyl-4-aminopyrimidyl (5))-acetamide	1×10^{-5}	0
6. 2-Methyl-4-hydroxy-5-aminomethylpyrimidine	2×10^{-6}	0
7. 2-Methyl-4-hydroxy-5-hydroxymethylpyrimidine	2×10^{-6}	0
8. 2:5-Dimethyl-4-aminopyrimidine	1×10^{-5}	0
9. 2:6-Dimethyl-4-hydroxy-5-aminopyrimidine	1×10^{-5}	0
10. 2-Methyl-4-hydroxy-6-aminopyrimidine	1×10^{-5}	0
11. 2-Methyl-4-mercaptopyrimidine	1×10^{-5}	0
12. 2-Hydroxy-4-aminopyrimidine (cytosine)	2×10^{-5}	0

* The apparent activity of No. 4, 2-methyl-4-methoxy-5-aminomethylpyrimidine, was undoubtedly due to contamination with the corresponding 4-amino compound (No. 1, see p. 1243). The specimen was an I.G. Farbenindustrie product.

Nos. 1, 3, 6, 7: obtained from Drs A. R. Todd & F. Bergel.

Nos. 2, 4, 5, 9: obtained from I.G. Farbenindustrie and made by Andersag & Westphal [1937]; transmitted by Dr A. Lwoff (Institut Pasteur, Paris).

Nos. 8, 10, 11: obtained from Hoffmann-La Roche; transmitted by Dr A. Lwoff.

No. 12: synthesized by Dr G. M. Richardson [1936].

does affect the growth activity, this is evidence that the 5-CH_2— group is concerned in whatever function the pyrimidine has. The relative effects of the differently substituted 5-CH_2— group and the unsubstituted 5-CH_3 group probably indicate that the pyrimidine and thiazole are joined by *Staph. aureus*, this being possible with —CH_2NH_2, —CH_2OH, more difficult with —$CH_2NH.CSH$, but not possible with —$CH_2CO.NH_2$ or —CH_3. On chemical grounds the formation of the thiazolium compound is likely with the first three groupings; the corresponding 5-CH_2Br— pyrimidine is used in the Williams & Cline [1936] synthesis. It is not likely with —CH_3 and hydrolysis of

—$CH_2CO.NH_2$ would yield —CH_2COOH, having one carbon atom too many for the link.

Hypotheses suggesting that an active pyrimidine and thiazole are used by *Staph. aureus* separately for different reactions, or for different parts of the same process, with the corollary that aneurin itself is split into its two components seem improbable in the light of these findings. Hills [1938], who showed that aneurin or the corresponding pyrimidine and thiazole together were necessary for the normal metabolism (aerobic or anaerobic) of pyruvate by *Staph. aureus*, found that the maximal effect of adding the two components simultaneously appears slightly later than the full effect of adding aneurin in a parallel experiment, suggesting a short lag while aneurin was made by linking the components. Other reasons for believing that the complete molecule or derivatives of it, are the active substances required for essential metabolic reactions in *Staph. aureus*, are discussed later.

Thiazoles

The most interesting new features shown by the thiazoles now examined are the reduced but real potencies of the β- and γ-hydroxypropyl compounds (Nos. 9 and 10, Table III), the delayed effect with the vinyl compound (No. 14, Table III), and the activity of 4-methyl-5-β-acetoxyethylthiazole (No. 2, Table III). Together

Table III. *Thiazoles*

		Conc. M	Growth 24 hr.
1.	4-Methyl-5-β-hydroxyethylthiazole	5×10^{-8}	+ + + +
		1×10^{-6}	+ + + +
		2×10^{-9}	+ +
		4×10^{-10}	tr.
2.	4-Methyl-5-β-acetoxyethylthiazole	1×10^{-6}	+ + +
3.	4-Methyl-5-α-hydroxyethylthiazole	5×10^{-7}	0
4.	3-Benzyl-4-methyl-5-β-hydroxyethylthiazole*	1×10^{-6}	+ + +
		1×10^{-7}	+ +
		1×10^{-8}	tr.
5.	3-(4'(5')-Methylimidazole)-4-methyl-5-β-hydroxyethylthiazole*	1×10^{-6}	+ + +
		1×10^{-7}	+ +
		1×10^{-8}	+
6.	2:4-Dimethyl-5-β-hydroxyethylthiazole	5×10^{-7}	0
7.	2-Hydroxy-4-methyl-5-β-acetoxyethylthiazole	1×10^{-5}	0
8.	4-Methyl-5-ethylthiazole	5×10^{-7}	0
9.	4-Methyl-5-β-hydroxypropylthiazole	5×10^{-7}	+ +
		5×10^{-8}	0
10.	4-Methyl-5-γ-hydroxypropylthiazole	5×10^{-7}	+
		5×10^{-8}	0
11.	4:5-Dimethylthiazole	1×10^{-7}	0
12.	4-Methylthiazole	1×10^{-5}	0
13.	4-Methyl-5-mercaptothiazole	1×10^{-5}	0

			22 hr.	43 hr.	90 hr.
14.	4-Methyl-5-vinylthiazole	$5 \times 10^{-7} M$	0	tr.	+
		$5 \times 10^{-8} M$	0	0	0

* Nos. 4 and 5. The apparent activity of these two compounds was probably due to contamination with the correct thiazole (i.e. not substituted at 3) from which they would be prepared (see text, p. 1246).

Nos. 1 and 12. Synthesized by Drs A. R. Todd & F. Bergel.

Nos. 2 and 7: I.G. Farbenindustrie product [Andersag & Westphal, 1937]; transmitted by Dr Lwoff.

Nos. 4, 5, 11 and 13: Hoffmann-La Roche product; transmitted by Dr Lwoff.

Nos. 3, 6, 8, 9, 10 and 14: obtained from Dr E. R. Buchman (California Institute of Technology, Pasadena, U.S.A.).

these show that there is some, although limited, scope for variation in the structure of the thiazole which is an essential part of the growth requirements oı *Staph. aureus.*

The activity of 4-methyl-5-β-acetoxyethylthiazole is not unexpected; conversion into the aneurin thiazole (5-β-hydroxyethyl) merely involves hydrolysis of the acetate. *Staph. aureus* is known to be able to hydrolyse an ester link, as in methyl nicotinate (see Table IV).

We are assured by Dr E. R. Buchman, who supplied us with the specimens of 4-methyl-5-β- and -γ-hydroxypropylthiazoles that the activities we observe are not due to traces of contaminating active impurity (the aneurin thiazole). He and his colleagues had occasion to examine this point with regard to *Phycomyces* growth activity (see below) and found that these hydroxypropyl derivatives *plus* the correct pyrimidine had activities equal to the corresponding aneurin analogues which had been recrystallized several times. The quantitative equivalence found could not therefore be explained by an active impurity in these two cases. We feel confident that tests of the corresponding aneurin analogues on *Staph. aureus* will confirm the real but much lower potency (e.g. 1/1000) of the 4-methyl-5-β- and -γ-hydroxypropylthiazoles found in the present tests. The hydroxypropyl aneurin analogues had no antineuritic activity in rats at a dose 1000 times the curative dose of aneurin (Dr E. R. Buchman; private communication).

The delayed activity of the 4-methyl-5-vinylthiazole (No. 14, Table III) is real; an active contamination would give an immediate response. It is reasonable to suppose that some of the active 5-β-hydroxyethyl compound is formed by addition of water to the —CH=CH$_2$ side chain. The effect with the vinyl compound is presumably delayed while this takes place.

The inactivity (no effect at 1 to $2 \times 10^{-7} M$) of the 4-methyl-5-α-hydroxyethyl-, 4-methyl-5-ethyl- and 4:5-dimethyl-thiazoles, and the lowered potency of the hydroxypropylthiazoles (an effect at $5 \times 10^{-7} M$) compared with the activity of the aneurin thiazole (4-methyl-5-β-hydroxyethylthiazole) which shows a detectable effect at $10^{-9} M$, illustrates the importance of the side chain at position 5.

It is also interesting that substitution of a —CH$_3$ or —OH group at position 2 in the thiazole ring (Nos. 6 and 7, Table III) inactivates an otherwise active thiazole; the same holds for a corresponding aneurin analogue (2-methylaneurin). When aneurin is oxidized to thiochrome it is the C atom at 2 in the thiazole ring which links with the nitrogen in the 4-amino group in the pyrimidine. The inactivity of these 2-substituted thiazoles suggests the question whether oxidation to thiochrome is involved in any of the metabolic functions of aneurin.

The apparent activity of the two N-substituted compounds, Nos. 4 and 5, Table III, cannot be accepted as real without examination of further purified specimens, since the method of preparation, from the highly active aneurin thiazole itself, renders contamination by the latter very probable. Furthermore similar pure quaternary salts have been found inactive in the *Phycomyces* growth test, which in general parallels the growth effects with *Staph. aureus* (E. R. Buchman, private communication).

Aneurin and its pyrimidine and thiazole components in the growth of various organisms

The observation that the pyrimidine and thiazole components of aneurin together were as effective in the growth of *Staph. aureus* as aneurin itself [Knight, 1937, 3] has been extended to other micro-organisms. It has been shown that the corresponding thiazole-pyrimidine pairs are effective in the growth of the

mould *Phycomyces blakesleeanus* [Schopfer & Jung, 1937; Schopfer, 1937; Sinclair, 1937; Robbins & Kavanagh, 1937; 1938]. Similarly Lwoff & Dusi [1937; 1938], Lwoff, A. & Lwoff, M. [1937; 1938] and Lwoff, M. [1937] have extended the field of action of aneurin or its components by showing that they are essential nutrients for certain unicellular protista [*Eucaryotes*; Lwoff, 1932].

A comparison of the need for aneurin or its components in a wide group of fungi has been made by Robbins [1938] and Robbins & Kavanagh [1938, 3], for species of the genus *Ustilago* by Schopfer & Blumer [1938], and for *Mucor Ramannianus* (Möll) by Müller & Schopfer [1937].

These investigations have shown that there are some species which need be given only one or other of the aneurin components, while other species cannot utilize the two components but must be given the complete aneurin molecule. Of the latter type are the flagellate trypanosome *Strigomonas oncopelti* [Lwoff, M. 1937]; the ciliate *Glaucoma piriformis* [Lwoff, A. & Lwoff, M. 1937, 2; 1938] and *Ustilago scabiosae* (Sow.) [Schopfer & Blumer, 1938]. Aneurin analogues inactive for *Staph. aureus* were also inactive for *Glaucoma piriformis* [Lwoff, A. & Lwoff, M. 1938].

All the strains of *Staph. aureus* examined in our laboratory (now about 30 typical strains) have required both the pyrimidine and the thiazole. In no case has a strain been observed which needed only one of them, or which could utilize only aneurin and not the two components. It is possible that other species of bacteria may be found which exhibit these growth requirements.

We are indebted to Dr G. P. Gladstone for the unpublished observations that an examination of the filtrates from cultures of *Chromobact. prodigiosum*, *Pseudomonas pyocyanea*, *B. subtilis* and *Bact. typhosum*, has shown that all these bacteria, cultivated in media which do not contain aneurin, do in fact synthesize this substance (or its two components) since the filtrates can supply the aneurin requirements of *Staph. aureus*. This is an extension of the earlier observation of Hughes [1932] with *Bact. coli*. For these bacteria therefore the dispensability of aneurin (or its components) as a nutrient essential does not mean that this substance may not be involved in their metabolism; it is in fact synthesized by these organisms.

Robbins & Kavanagh [1938, 3] found that those species of fungi which required only one or other aneurin component for growth synthesized the complementary component, since the culture filtrates could supply both components to *Phycomyces blakesleeanus*. Analogous observations for *Mucor Ramannianus* and *Rhodotorula* were made by Müller & Schopfer [1937] each organism supplying one component to the other. In general therefore it is found for the organisms discussed here that a need for one or both aneurin components, or for aneurin itself, as nutrients, reflects the relative ability of the organism to synthesize aneurin. The latter appears to be required in the metabolism of all of them, and it or its components are nutrient essentials if they cannot be synthesized.

An examination of the specificity of pyrimidines and thiazoles which can be used by various fungi and unicellular protista in place of the aneurin which provides essential nutrient material for them, and a comparison with similar observations for *Staph. aureus*, supports this view. Generally speaking the specificity in the growth of *Staph. aureus* is paralleled by the effects shown by *Phycomyces* [Robbins & Kavanagh, 1938, 1, 2; Schopfer, 1937; Sinclair, 1937] and by the protista studied by Lwoff & Dusi [1938, 2, 3]. It will therefore be simplest to note only the points of difference.

(a)   *2-Methyl-4-amino-5-thioformamidomethylpyrimidine*.   Sinclair [1937] found for *Phycomyces blakesleeanus* that this compound was less potent than the

corresponding 5-aminomethyl derivatives, in agreement with the results with *Staph. aureus*. Robbins & Kavanagh [1938, 1] confirmed this, using a sample of the 5-thioformamidomethyl derivative from the same source as the specimens used by Sinclair and by Knight, namely Dr A. R. Todd. Further, Robbins & Kavanagh showed that the activity of the 5-thioformamidomethyl derivative decreased on autoclaving to sterilize, a filtered solution being the most active. This did not occur with the 5-hydroxymethyl or 5-bromomethyl derivatives. Presumably it would not occur with the 5-aminomethyl derivative which would be the probable active contaminant of the 5-thioformamidomethyl compound if one were present (this follows from the mode of synthesis [Todd & Bergel, 1937]). Schopfer [1937] with a different specimen of the 5-thioformamidomethyl derivative (Hoffmann-La Roche) recorded only very feeble activity for *Phycomyces*, but he used an autoclaved solution. Lwoff & Dusi [1938, 2] found that a specimen of the 5-thioformamidomethyl derivative (also from Hoffmann-La Roche) but not autoclaved, had activity for *Polytomella caeca* and *Chilomonas paramoecium*, but was less potent than the corresponding 5-aminomethyl compound. With the exception of Schopfer's results, probably explicable by loss of activity on autoclaving, all the observations are in agreement that the thioformamidomethyl derivative can be utilized by *Staph. aureus*, *Phycomyces blakesleeanus*, *Polytomella caeca* and *Chilomonas paramoecium*, but in all cases is less readily available than the corresponding 5-aminomethyl, bromomethyl or hydroxymethyl derivative. This suggests a common objective in the utilization of the pyrimidine by all these organisms.

(b) *Thiazoles*. The most striking difference of behaviour in the utilization of thiazoles by the different organisms considered here is that shown with the compound 2:4-dimethyl-5-β-hydroxyethylthiazole. This was inactive for *Staph. aureus* (No. 6, Table III) and for *Phycomyces blakesleeanus* [Robbins & Kavanagh, 1938, 2]; but Lwoff & Dusi [1938, 3] using the same specimen as we have used in the present work find that it can be utilized by the flagellates *Polytoma caudatum*, *P. ocellatum*, and *Polytomella caeca*. The corresponding 2-hydroxy-4-methyl-5-β-acetoxyethylthiazole was inactive for these flagellates as it was for *Staph. aureus* (No. 7, Table III).

With the exception of this difference the specificity of the pyrimidine and thiazole components of aneurin is the same for all the organisms discussed here. These results emphasize the high specificity of the aneurin structure in the physiology of all these varied types of organism and also harmonize with the results obtained by Bergel & Todd [1937] with aneurin analogues for antineuritic activity in the rat, suggesting a fundamental metabolic role for aneurin common to all these organisms.

Compounds related to nicotinamide

The results collected in Table IV emphasize the specificity of nicotinamide and two closely related compounds in the growth of *Staph. aureus*. One of the functions of the nicotinamide must be to contribute that molecule to the synthesis of codehydrogenase I or II (pyridine nucleotide phosphate compounds) which *Staph. aureus* is known to produce. This follows from the fact that the organism is known to synthesize the " *V* factor " for *Haemophilus parainfluenzae*, which Lwoff, A. & Lwoff, M. [1937, 1] have shown to be identical with either codehydrogenase I or II. Nicotinamide is more active than nicotinic acid, in agreement with the fact that it is the amide which is required in the codehydrogenase. *Staph. aureus* can make the amide from the acid if necessary. It appears therefore that *Staph. aureus* must find nicotinic acid preformed be-

Table IV. *Pyridine derivatives*

	Conc. M	Growth 24 hr.
1. Nicotinamide	2×10^{-7}	+ + + +
	4×10^{-8}	+ +
	8×10^{-9}	?
2. Pyridine-3-carboxylic acid (nicotinic acid)	1×10^{-6}	+ + +
	2×10^{-7}	?
3. Methyl nicotinate	5×10^{-7}	+ (3 days)
	1×10^{-7}	? (3 days)
4. Pyridine-3-carboxylic acid diethylamide ("Coramine")	1×10^{-5}	0
5. Pyridine-3-sulphonate	1×10^{-1}	0
6. Pyridine-3-nitrile	2×10^{-5}	0
7. 3-Methylpyridine (β-picoline)	1×10^{-4}	0
8. Trigonelline methyl sulphate	1×10^{-4}	0
9. Trigonelline chloride	1×10^{-4}	0
10. Pyridine-4-carboxylic acid (*iso*nicotinic acid)	1×10^{-4}	0
11. Pyridine-2-carboxylic acid (picolinic acid)	1×10^{-4}	0
12. Pyridine-2:3-dicarboxylic acid (quinolinic acid)	1×10^{-4}	0
13. 2:4-Dimethylpyridine-3:5-dicarboxylic acid	1×10^{-4}	0
14. 2:4:6-Trimethylpyridine-3:5-dicarboxylic acid	1×10^{-4}	0
15. Nicotine	1×10^{-5}	0

None of the compounds which failed to permit growth was toxic, since growth ensued on subsequently adding nicotinamide. Failure of growth was therefore due to non-utilizability.

No. 4. "Coramine" was obtained from Messrs Ciba, Ltd., through the courtesy of Dr K. Miescher.

Nos. 6 and 9: obtained from Dr H. King.

Nos. 10 and 12: obtained from Prof. G. R. Clemo.

No. 2. A commercial specimen of nicotinic acid which even on repeated crystallization from water gave pale yellow warty clusters of needles, was obtained free from the persistently associated impurities by sublimation and recrystallization from water after digestion with norite charcoal. It then separated in long colourless flat needles, m.p. 232° (uncorr.).

No. 5. *Pyridine-3-sulphonic acid* was prepared by a method based on those given by Meyer & Ritter [1914] and Gastel & Weiband [1934].

No. 8 *Trigonelline methyl sulphate.* Nicotinic acid (1·0 g.) and freshly distilled dimethyl sulphate (0·8 ml.) heated at 120 for 15 min., yielded a viscous gum which when crystallized from alcohol gave colourless prisms of the methosulphate, m.p. 210 . The alcoholic solution with picric acid yielded trigonelline picrate, m.p. 204 .

No. 13. *2:4-Dimethylpyridine-3-carboxylic acid hydrochloride* was prepared according to Michael [1885].

No. 14. *2:4:6-Trimethylpyridine-3:5-dicarboxylic acid* (K salt) was prepared according to Hantzsch [1882].

No. 11. The copper salt of pyridine-2-carboxylic acid was obtained from Prof. G. R. Clemo, and the free acid (m.p. 139°) was prepared from it by passing H_2S over the dry salt and extracting with water.

Nos. 7 and 15 were both commercial specimens.

cause it cannot synthesize it: the remainder of the codehydrogenase molecule (ribose, adenine) it can synthesize. The inactivity of the diethylamide ("coramine") indicates that the organism cannot remove the two ethyl groups. It can hydrolyse the methyl ester of nicotinic acid since methyl nicotinate is active, although growth is delayed. The other compounds in Table IV, all of which are inactive, emphasize how small an alteration from the structure appearing in the codehydrogenase molecules produces inactivity in growth.

Nicotinic acid has been found among the nutrient requirements of strains of *C. diphtheriae* by Mueller [1937] and of strains of dysentery bacilli by Koser *et al.* [1938]. Fildes [1937] has indicated a series of bacteria whose relative abilities to synthesize the whole or parts of the codehydrogenase 1 or II molecules are reflected in the nutrient requirements of the different species. It appears probable that the organisms studied by Mueller and by Koser *et al.* will be found to synthesize codehydrogenase I or II, or both.

SUMMARY

An examination of a further series of aneurin and pyridine derivatives emphasizes the previous conclusion that aneurin and nicotinamide are highly specific in the growth requirements of *Staph. aureus*. Only very limited departures from these structures are permissible if growth activity is to be maintained.

A comparison with similar observations made upon other types of organisms, in particular certain fungi and unicellular protista, suggests an underlying biochemical unity.

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Note added 8 June 1938. Since the above was written Woolley, Strong & Madden (*J. biol. Chem.* 1938, **123**, *Proc.* cxxxi) have reported a similar high specificity of structure for pyridine derivatives in the cure of canine black-tongue. They had previously shown that nicotinic acid or its amide could effect the cure (Elvehjem, Madden, Strong & Woolley, *J. Amer. chem. Soc.* 1937, **59**, 1767; *J. biol. Chem.* 1938, **123**, 137). The non-utilisable derivatives agree with those found for *Staph. aureus* with the exception that β-picoline has a definite effect, and nicotinic acid N-methylamide a slight effect with the dogs.

CLXVII. A PHYSICO-CHEMICAL AND BIOCHEMICAL STUDY OF VITAMIN A₂

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THE literature concerning the existence of different substances in fish liver oils which give a colour with antimony trichloride has been reviewed recently in detail in this *Journal* by Edisbury *et al.* [1938] and by Gillam *et al.* [1938]. These investigators have established the existence in freshwater fish liver oils of a higher homologue of vitamin A, the "693 mμ chromogen" or "vitamin A₂".

Full details are given below of experiments reported briefly elsewhere [Lederer & Rathmann, 1938], which show that vitamin A₂ gives in the presence of $SbCl_3$ a second absorption band not previously observed. This band is of importance for the calculation of the relative quantities of vitamins A₁ and A₂ in any given material. Our paper deals further with data obtained from oils of freshwater fish from different parts of Europe as well as from oils or fats from other animals. Experiments dealing with the absorption of vitamin A₂ from the intestine and its accumulation in the liver of the rat and the frog are included.

EXPERIMENTAL

Unsaponifiable fractions were prepared by the usual methods [cf. Gillam *et al.* 1938]. The livers and intestines were hydrolysed with aqueous or alcoholic NaOH (5–10%) and extracted with ether; the resulting concentrate was saponified. As the alkaline hydrolysis was always accompanied by a partial saponification of the oil, no values for the oil content were available. In Tables I and III are given the weights of the unsaponifiable fractions calculated for 100 g. of fresh organ. All measurements were made on the unsaponifiable fractions. For the determination of absorption coefficients a Hilger-Nutting visual spectrophotometer was used. All readings were repeated at least twice by one of us and controlled by the other. In Tables I and III it was thought sufficient to give the data for $E_{1\,cm.}^{1\%}$ 695 mμ, as the corresponding values for 650 and 620 mμ can be easily calculated from the ratios E 695 mμ/E 650 mμ and E 695 mμ/E 620 mμ given in the tables.

The inhibitor (Table III, no. 18) was prepared as follows. After saponification of the pike liver oil (Table III, no. 17) the ether-extracted soap was acidified with 5% H_2SO_4 and extracted three times with ether. The solvent was dried with Na_2SO_4 and distilled off. The colourless residue crystallized incompletely. 20 mg. dissolved in 0·1 ml. $CHCl_3$ were added to 0·2 ml. of a solution of the pike unsaponifiable no. 17 of Table III.

The 650 mμ band of vitamin A₂

There is evidence that vitamin A₂ is a higher homologue of vitamin A₁, having six conjugated double bonds, a ring of β-ionone and one hydroxyl group [Gillam *et al.* 1938]. It would be surprising if this substance had only one absorption band in presence of $SbCl_3$, seeing that vitamin A₁ and other natural

carotenoids with hydroxyl groups (zeaxanthin, lutein, violaxanthin) show two bands [Castle *et al.* 1934; Gillam, 1935: Euler & Karrer, 1932; Euler *et al.* 1932]. The hydrocarbons (α- and β-carotene, lycopene and *iso*carotene) show only one band [Karrer & Walker, 1933; Gillam, 1935]. This is also true for synthetic polyenes. Thus the tetradecapentaen-ol of Kuhn & Grundmann [1938] gives two bands with SbCl₃, whereas the tetradecahexaene prepared from the former by splitting off water gives only one band. The *apo*carotinols of Euler *et al.* [1938] seem to be exceptions to these rules.

We have found that vitamin A₂ has indeed a second band (situated at 645–650 mμ) but this band is usually masked, just as is the second band of vitamin A₁ at 580 mμ; it can be made visible by adding an inhibitor, in the same way as can the second band of vitamin A₁ [Emmerie *et al.* 1931; Eekelen *et al.* 1931; Castle *et al.* 1934].

Table III shows the intensity of absorption at 695 mμ of concentrates from fish liver and intestines. For those which have a relative absorption intensity E 695 mμ/E 620 mμ higher than 2, the intensity of absorption at 650 mμ is about two-thirds of that at 695 mμ, the quotient E 695 mμ/E 650 mμ varying from 1·38 to 1·80. This variation is probably not significant, so that it may be concluded that both bands are due to the same substance (see Fig. 1).

The 650 mμ band can be clearly seen on adding to the solution of the unsaponifiable in chloroform the fraction of fatty acids obtained after saponification of freshwater fish liver oils. The absorption at 695 mμ is much diminished, while absorption at 650 and at 620 mμ remains almost unchanged (Table III, nos. 17, 18).

The band at 640 or 645 mμ observed in some natural oils [Heilbron *et al.* 1931; Morton, 1932; Edisbury *et al.* 1938: Gillam *et al.* 1938] seems to correspond to the 645–650 mμ band of vitamin A₂. After saponification of these oils the inhibitor is removed and the 693 mμ band reaches its full strength and masks the second band (cf. Gillam *et al.* [1938], oil of *Huso huso*). The same band is seen in partially saponified oils together with the 693 mμ band and is then located at 645 mμ. On the other hand, the 645 mμ band is sometimes observed in partially decomposed vitamin A₂ concentrates [Gillam *et al.* 1938]; its appearance is due in these cases also to a diminution of the intensity of the 693 mμ band.

In some natural oils full inhibition of the 693 and 650 mμ bands occurs and such oils show only a weak band at 610 mμ (Table III, no. 2). After complete saponification the 610 mμ band disappears, overshadowed by the strong 693 mμ band.

Quantitative determination of vitamins A₁ and A₂

Until quite recently the determination of the relative quantities of vitamins A₁ and A₂ present in an oil was based on two assumptions: (1) that $E_{1\,cm}^{1\,\%}$ 693 mμ for vitamin A₂ has the same value as $E_{1\,cm}^{1\,\%}$ 620 mμ for vitamin A₁, i.e. about 5000 [Edisbury *et al.* 1938; Gillam *et al.* 1938]; (2) that the absorption at 620 mμ is due solely to vitamin A₁ and that at 693 mμ solely to vitamin A₂.

The first assumption seems to be fairly correct as shown by the following consideration. Vitamin A₂ having one conjugated double bond more than vitamin A₁, its molecular extinction coefficient at 345 mμ should be somewhat higher (about 20 %) than that of vitamin A₁ at 328 mμ. Since, on the other hand, a 1 % solution of vitamin A₂ contains about 10 % fewer molecules than a 1 % solution of vitamin A₁, the $E_{1\,cm}^{1\,\%}$ values should be about the same for both in the ultraviolet. Moreover, solutions of vitamins A₁ and A₂ having equal extinction at 328 and 345 mμ respectively show roughly the same $E_{1\,cm}^{1\,\%}$ at 620

and 693 mμ, as illustrated by the following figures for vitamin A_2 taken from Gillam et al. [1938].

Vitamin A_2 $E_{1\,cm.}^{1\,\%}$ 344 mμ = 850 $E_{1\,cm.}^{1\,\%}$ 697 mμ = 2600

Vitamin A_1 $E_{1\,cm.}^{1\,\%}$ 328 mμ = 850 $E_{1\,cm.}^{1\,\%}$ 620 mμ = 2650 (calculated on the basis of the usual E values for A_1)

The second assumption is unjustified in so far as it concerns absorption at 620 mμ, for this has been shown to be only partly due to vitamin A_1 [Lederer & Rathmann, 1938].

On measuring the absorption intensities at 620 and 580 mμ of vitamin A_1 concentrates as far as possible free from vitamin A_2 (E 695 mμ/E 620 mμ < 0·17) it was found that E 650 mμ/E 620 mμ for these preparations was approximately 0·25–0·30 (Table I, nos. 2–5). A value of 0·35 was obtained by evaluation of the absorption curve of vitamin A from frog retina measured by Wald [1936].

Table I. *Vitamin A_1 from marine fish and land animals*

No.	Species	Organ	Wt. g.	Nonsap. mg. per 100 g. organ	$E_{1\,cm}^{1\,\%}$ 695 mμ	$\dfrac{E\ 650\ m\mu}{E\ 620\ m\mu}$	$\dfrac{E\ 695\ m\mu}{E\ 620\ m\mu}$
1	*Gadus merlangus*	8 livers	160	400	2·5	0·61	0·21
2	Halibut (*Hippoglossus hippoglossus*)	—*	—	—	40·0	0·29	0·17
3	*Ishinagi sterelepis*	—*	—	—	112·0	0·26	0·15
4	Sheep (*Ovis aries*)	1 liver	1080	430	1·5	0·27	0·03
5	Chick (*Gallus domesticus*)	1 liver	27	410	1·2	0·35	0·08
6	Frog (*Rana esculenta*)	14 livers	11	600	8·2	0·52	0·27

* Oil investigated.

If one supposes that the absorption curve of pure vitamin A_2 falls off at the same rate from 650 to 620 mμ as the absorption curve of vitamin A_1 from 620 to 650 mμ, one reaches the conclusion that E 620 mμ/E 650 mμ for pure vitamin A_2 must also be about 0·25–0·30.

The relative intensities of absorption of our richest vitamin A_2 concentrates were: E 695 mμ/E 650 mμ/E 620 mμ = 3/1·8/1. Now if E 620 mμ/E 650 mμ for vitamin A_2 is 0·25, then 0·45 (i.e. 1·8 × 0·25) or nearly half of the absorption at 620 mμ is due to vitamin A_2.

On the basis of these figures we suppose that E 695 mμ/E 620 mμ for pure vitamin A_2 will be between 6 and 8. (The values previously reported by Lederer & Rathmann [1938] were too low.) Taking 7 as an average value, the relative quantities of vitamins A_1 and A_2 may be calculated by the formula

$$\text{vitamin } A_2/\text{vitamin } A_1 = E\ 695\ m\mu \Big/ \left(E\ 620\ m\mu - \frac{E\ 695\ m\mu}{7} \right);$$

Table II gives the relative quantities of the two vitamins calculated directly from the ratio E 695 mμ/E 620 mμ.

Fig. 1 shows the absorption curve of a vitamin A_2 concentrate (E 695 mμ/ E 620 mμ = 2·5) in presence of antimony trichloride. The crosses indicate average values from measurements on 10 different unsaponifiable fractions taken from different species of fish with ratios varying from 2·3 to 2·7. The measured curve can be shown to result from 4 separate hypothetical absorption curves with maxima at 693, 650, 620 and 580 mμ respectively (light lines). The figure demonstrates clearly the overlapping of the absorption bands of vitamins A_1 and A_2.

Table II. *Relative quantities of vitamins A_1 and A_2*

$\dfrac{E\ 695\,m\mu}{E\ 620\,m\mu}$	$\dfrac{\text{Vitamin A}_2}{\text{Vitamin A}_1}$	% vitamin A$_2$	% vitamin A$_1$
0·5	0·54	35	65
1·0	1·16	54	46
1·5	1·88	65	35
2·0	2·80	74	26
2·5	3·90	80	20
3·0	5·30	84	16
4·0	9·30	91	9
5·0	17·00	95	5
6·0	40·00	98	2
7·0	∞	100	0

Of course these figures have to be considered as a first approximation and will have to be revised as soon as more exact values are available. They are independent of the chemical structure of vitamin A$_2$, which seems to need further investigation, as pointed out by Euler *et al.* [1938].
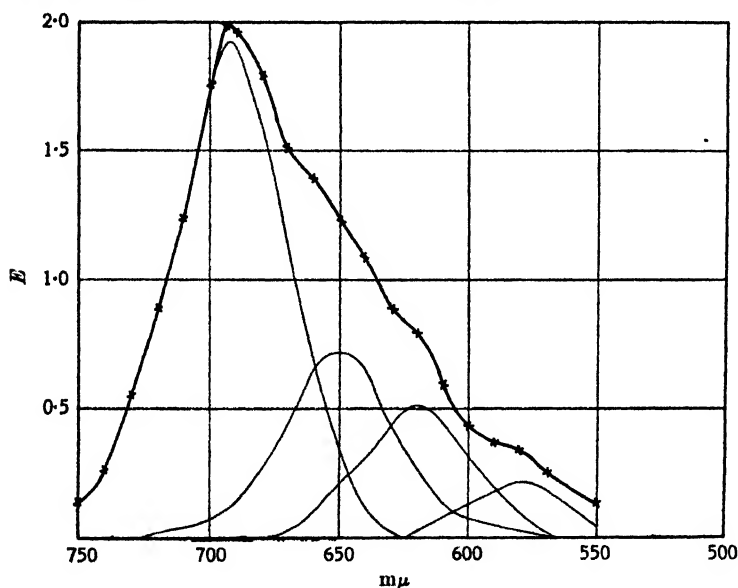
Fig. 1. Absorption spectrum of vitamin A$_2$ concentrates ($E\,695\,m\mu/E\,620\,m\mu = 2\cdot5$) in presence of SbCl$_3$ (× — × — ×). The light lines show the four hypothetical bands with maxima at 695, 650, 620, 580 mμ.

Our results are of importance in considering the biological assay of vitamin A$_2$ reported by Gillam *et al.* [1938]. A final conclusion as to the activity of vitamin A$_2$ was difficult to draw at that time as the assayed preparation was thought to contain only two parts of vitamin A$_2$ to one part of vitamin A$_1$ ($E\,695\,m\mu/E\,620\,m\mu = 2$). Since the activity of this concentrate was somewhat less than that of a halibut oil fed in equal doses, it could have been argued that the activity was due solely to the vitamin A$_1$ present. As the value of 2 for the ratio $E\,695\,m\mu/E\,620\,m\mu$ had been measured more than 2 weeks after the preparation of the oil, we suppose that the real value for the fresh oil was at least 2·3, as for all *Lucioperca lucioperca* concentrates measured immediately after preparation (*vide infra*). In the light of the conclusions reached above the assayed preparation contained only about 20–25 % vitamin A$_1$. This seems to prove that the 693 mμ chromogen deserves the name vitamin A$_2$ (cf. the critical remarks

of Euler *et al.* [1938]). Exact assays of our preparations have now been begun by Dr Th. Moore to determine more precisely the biological activity of vitamin A_2.

Ultraviolet absorption spectra of vitamin A_2 concentrates

Gillam *et al.* [1938] as well as Edisbury *et al.* [1938] stated that vitamin A_2 concentrates have two absorption bands in the ultraviolet, one at $345 \, m\mu$ and the other at $280 \, m\mu$. The former authors are inclined to think that both bands are due to vitamin A_2 because of the more or less constant relative intensities of the two bands in concentrates from different sources, whereas Edisbury *et al.* seem to have separated the two bands by treating vitamin A_2 concentrates with 83% alcohol. The insoluble fraction is shown to have the band at $280 \, m\mu$ whereas the soluble fraction, representing vitamin A_2, has a band at $345 \, m\mu$. Edisbury *et al.* [1938] suppose that the insoluble fraction is identical with that isolated before by Pritchard *et al.* [1937] from a mammalian concentrate in the same way and that it represents a lower (C_{18}) homologue of vitamin A_1.

These conclusions seem rather improbable, because it is difficult to believe that the C_{18} homologue of vitamin A_1 should be quite insoluble in 83% ethyl alcohol in which vitamin A_1 itself is easily soluble. Moreover, in our experiments we were unable to confirm the findings of Edisbury *et al.* concerning the separation of the two bands in the ultraviolet.

2·2 g. of a concentrate from 50 pike livers (Table III, no. 14) were dissolved in methanol and freed from sterols (1·6 g.) by freezing at - 12°. The filtrate was then evaporated to dryness *in vacuo* and dissolved in 83 % ethyl alcohol; after standing for an hour at - 12° an amorphous precipitate settled out which was filtered off. The filtrate was evaporated to dryness *in vacuo* and the residue was completely soluble in 77 % ethyl alcohol at room temperature. After standing for an hour at - 12° a second (oily) precipitate could be separated. The filtrate contained practically the whole of the vitamin A_2 (320 mg. with $E_{1 \, cm}^{1 \, \%}$ 695 mμ = 1060) and showed two bands in the ultraviolet (345 and 280 mμ) with relative intensities practically the same as those of the original material, as far as could be judged from the photographs taken with an Yvon and Jobin spectrograph of small dispersion.

It seems therefore that both bands in the ultraviolet belong to vitamin A_2. It may be mentioned here that Gillam [1935] found a $268-280 \, m\mu$ band in the ultraviolet part of the absorption spectrum of all the carotenoids which he investigated.

The alcohol treatment resulted in a slight increase of the ratio $E \, 695 \, m\mu / E \, 620 \, m\mu$, i.e. from 2·65 for the original material (Table III, no. 14) to 3·0 for the fraction soluble in 77 % ethyl alcohol. The insoluble fraction had a ratio of 2·0.

Colour reactions of vitamins A_1 and A_2

In the hope of finding some reagent which would enable one to distinguish more specifically between the two vitamins than is possible with $SbCl_3$, a number of other substances were studied. Rosenheim & Drummond [1925] reported that trichloroacetic acid, dimethyl sulphate, perchloric acid, benzoyl chloride, acetyl chloride and arsenic trichloride gave blue solutions in the presence of vitamin A, but they recorded no spectroscopic observations, except for arsenic trichloride.

The following table gives the results of measurements made on a marine and a freshwater fish liver concentrate ($E \, 695 \, m\mu / E \, 620 \, m\mu = 2·5$ for the latter).

Reagent	Vitamin A_1	Vitamin A_2	Remarks
Trichloroacetic acid	610 mµ	690 mµ	30% reagent in $CHCl_3$
Dimethyl sulphate	615 ,,	680 ,,	20% reagent in $CHCl_3$
Perchloric acid	605 ,,	680 ,,	5–10% reagent in $CHCl_3$
Benzoyl chloride	Very weak	590 ,,	20% reagent in $CHCl_3$
Acetyl chloride	572 mµ	No band	A_1, very unstable violet colour; A_2, first blue, then violet
Arsenic trichloride	628, 577 mµ	710, then 590 mµ	A_2, first green, then violet
Reaction of Rosenthal & Erdélyi [1934]	558, 480, 450 mµ	685 mµ	A_2, no colour change on heating

These results were not encouraging for further studies, as the bands were more or less the same as those which occur in the Carr and Price reaction.

Determination of the ratio E 695mµ/E 620mµ in liver oils of freshwater fish from different sources

A striking difference seemed to exist between the results of Edisbury et al. [1938] on English fish and those of Gillam et al. [1938] on Russian fish. The former authors found in general a ratio E 693 mµ/E 620 mµ varying from 0·8 to 1·5 (for the livers of *Salmo salar*, *Salmo trutta* v. *fario*, *Salmo irideus* and *Perca fluviatilis*), whereas the livers of Russian freshwater fish (*Lucioperca lucioperca, Esox lucius, Silurus glanis, Abramis brama, Huso huso, Stenodus leucichthys nelma, Coregonus lavaretus pidschian* etc.) had ratios varying from 1·6 to 2·6. Only a few varieties of salmon and carp had low values (0·25–0·88).

We decided to investigate this point and measured the relative absorption intensities at 695 and 620 mµ of liver concentrates from a number of fish from various parts of Europe (Austria, France, Holland, Hungary and Yugoslavia). The results showed (Table III) that there was no geographical difference but a very marked species specificity as regards these relative intensities. Specimens of pike-perch (*Lucioperca lucioperca*), pike (*Esox lucius*) and catfish (*Silurus glanis*) from all over Europe had a ratio of 1·9–2·7, whereas carp (*Cyprinus carpio*), trout (*Salmo irideus*), salmon (*Salmo salar*) and sturgeon (*Acipenser sturio*) had much lower values (0·4–0·8). Marine fish from the Atlantic coast of France (*Gadus merlangus*, *Hippoglossus hippoglossus*) and from Japan (*Ishinagi sterelepis*) had still lower values (0·17–0·21). These measurements agreed well with those of Edisbury et al. [1938] and of Gillam et al. [1938] on English and Russian fish and the "striking difference" between the results of these two groups of investigators was simply due to the fact that in the two laboratories different species of fish were used.

We have observed that the beginning of oxidation in concentrates of vitamin A_2 is indicated by a decrease of the ratio E 695 mµ/E 620 mµ, due probably to the more rapid destruction of vitamin A_2. Some of the lower values for this quotient reported in the literature ([Gillam et al. 1938] and Table III of this paper) may be explained in this way.

Comparison of liver oils and intestinal oils

Edisbury et al. [1938] made the interesting observation that the intestinal oils of some fish contained great quantities of vitamin A, comparable with those found in the liver. They also recorded measurements of the ratios vitamin A_1/vitamin A_2 in the intestinal oils of freshwater fish, and found that as a rule they were lower than in the corresponding liver oils.

Our measurements confirmed the findings of Edisbury et al. and showed that in some species (carp, Table III, nos. 30–33) E 695 mµ/E 620 mµ of the intestinal oils was even higher than the corresponding ratio in liver oils from the same fish.

Table III. *Vitamins A₁ and A₂ in liver and intestine of freshwater fish*

No.	Name of fish	Origin	Organ	Wt. g.	Unsap. mg. per 100 g. fresh organ	$E^{1\%}_{1\,\text{cm}}$ 695 mμ	$\dfrac{E\,695}{E\,650}$	$\dfrac{E\,695}{E\,620}$	Remarks
1	Pike-perch (*Lucioperca lucioperca*)	France (Saône)	5 livers	52	340	40	1·80	2·63	Gillam *et al.* [1938] for Russian fish $\dfrac{E\,695\,\text{m}\mu}{E\,620\,\text{m}\mu}=2\cdot18\text{-}2\cdot34$
2	„	„	5 livers	52	(8 g. oil)	0·5	1·00	0·58	Oil from no. 7 before saponification
3	„	„	4 intestines	180	220	16	1·27	1·60	Whole intestine with stomach and fat
4	„	„	14 livers	93	300	88	1·65	2·40	
5	„	„	7 caeca	47	430	78	1·80	2·25	Same fish
6	„	„	Exterior caecal fat from 7 caeca	110	600	5	1·18	1·00	
7	„	Austria (Bodensee)	10 stomachs	70	250	2	1·00	1·00	
8	„	„	1 liver	27	500	110	1·60	2·60	
9	„	„	1 intestine	56	300	115	—	2·35	Measured 2 months after preparation of unsap.
10	„	Hungary	1 liver	25	360	100	1·46	2·00	
11	„	Holland	1 liver	18	560	15	1·55	2·35	
12	Pike (*Esox lucius*)	France	7 livers	65	300	84	1·66	2·00	Measured 7 days after preparation of unsap.
13	„	„	7 intestines	125	420	8	1·30	1·80	
14	Catfish (*Silurus glanis*)	„	50 livers	440	530	180	1·56	2·65	Gillam *et al.* [1938] for Russian fish: $\dfrac{E\,695\,\text{m}\mu}{E\,620\,\text{m}\mu}=1\cdot90$
15	„	Austria (Danube)	4 livers	40	250	112	1·38	2·00	
16	„	„	3 intestines	120	230	24	1·48	1·88	Measured 13 days after preparation of unsap.
17	„	Holland	1 liver	12	610	185	1·54	2·45	
18	„	„	1 liver	12	610	55	0·43	0·58	Unsap. no. 17 + inhibitor
19	Catfish (*Silurus glanis*)	Austria (Danube)	2 livers	100	750	40	1·41	2·00	Gillam *et al.* [1938] for Russian fish: $\dfrac{E\,695\,\text{m}\mu}{E\,620\,\text{m}\mu}=2\cdot0$
20	Perch (*Perca fluviatilis*)	Hungary	3 intestines	210	360	7	1·28	1·64	
21	„	„	5 livers	75	670	156	1·54	2·20	
22	„	Holland	5 livers	30	675	32	1·52	2·20	
23	(*Tinca tinca*)	France	10 livers + intestines	8	520	35	1·52	1·52	Young fish
24	Bream (*Abramis brama*)	„	10 livers + intestines	5	460	14	—	1·40	Young fish
25	(*Alburnus alburnus*)	„	80 livers + intestines	75	175	18	1·13	1·25	Young fish
26	Trout (*Salmo irideus*)	„	10 livers + intestines	11	500	8	1·20	0·75	Edisbury *et al.* [1938] for English fish: $\dfrac{E\,695\,\text{m}\mu}{E\,620\,\text{m}\mu}=0\cdot8$
27	„	„	22 livers	31	530	9	1·05	0·54	
28	„	„	25 caeca	60	370	9	0·84	0·42	Same fish
29	„	„	25 intestines	126	220	4	0·87	0·44	
30	Carp (*Cyprinus carpio*)	„	1 liver	30	300	7	0·67	0·42	Gillam *et al.* [1938] for Russian fish: $\dfrac{E\,695\,\text{m}\mu}{E\,620\,\text{m}\mu}=0\cdot25$
31	„	Yugoslavia	1 intestine	30	360	3	—	0·42	
32	„	„	2 livers	100	260	20	0·95	0·50	
33	„	„	2 intestines	160	400	5	1·20	0·70	
34	„	Hungary	3 livers	190	380	32	1·10	0·49	
35	„	Holland	1 liver	13	685	105	0·95	0·50	
36	Sturgeon (*Acipenser sturio*)	France (Atlantic coast)	1 liver	400	260	78	1·00	0·60	Gillam *et al.* [1938] for Russian fish: $\dfrac{E\,695\,\text{m}\mu}{E\,620\,\text{m}\mu}=1\cdot32$
37	Salmon (*Salmo salar*)	Holland	1 liver	75	210	144	1·10	0·72	Edisbury *et al.* [1938] for English fish: $\dfrac{E\,695\,\text{m}\mu}{E\,620\,\text{m}\mu}=0\cdot8\text{-}1\cdot0$

More detailed determinations were made on the distribution of vitamins A_1 and A_2 in the intestinal tract of the pike-perch (*Lucioperca lucioperca*). The pyloric caeca of this fish are surrounded by great quantities of fat which can be separated mechanically from the caeca themselves. For stomach and exterior caecal fat $E\,695\,m\mu/E\,620\,m\mu$ was 1·0 (Table III, nos. 6, 7). The corresponding quotient for the caeca themselves and for the liver was 2·4 (Table III, nos. 4, 5).

This difference may be explained as follows. The vitamin A of the food is composed of about equal parts of vitamins A_1 and A_2 ($E\,695\,m\mu/E\,620\,m\mu = 1$ for the stomach contents). After absorption, a part of the fat of the food is deposited in the fat depots around the caeca, a process which leaves the ratio vitamin A_1/vitamin A_2 unchanged. In the liver and the caeca, on the other hand, there seems to be a constant coming and going of fat resulting in a greater accumulation of vitamin A_2 owing to its more unsaturated character. This would be in agreement with the observations of Zechmeister & Tuszon [1935, 1] that the more unsaturated carotenoids lycopene and capsanthine are more easily stored in human fat than carotene or lutein, as well as with the experiments of Thorbjarnarson & Drummond [1938] which show that fat leaving the liver may carry away some vitamin A.

In one experiment the caeca of a pike-perch were emptied of their contents and the caecal skin and the contents were analysed separately, with the following results:

	Wt. g.	Nonsap. mg.	$E^{1\%}_{1\,cm.}$ 695 mμ	$E\,695\,m\mu/$ $E\,620\,m\mu$	Mg. vit. A_2 per 100 g.*
Caecal skin	2·1	27†	50·5	2·4	12·5
Caecal contents	1·9	12†	24·0	2·1	3·4

* Calculated on the assumption that $E^1_{1\,cm}$ 695 mμ of pure vitamin A_2 is 5000.
† Not entirely saponified.

The pyloric caecal skin had a high absolute content of vitamin A_2 which was more than twice as great as that of the corresponding liver (Table III, no. 4; 5·3 mg. vitamin A_2 per 100 g. fresh organ). It is reasonable to suppose that the accumulation of this vitamin in the caeca is not fortuitous but is related to the mechanism of fat absorption discussed by Edisbury *et al.* [1938] in connexion with vitamin A_1.

$E\,695\,m\mu/E\,620\,m\mu$ of liver fat of animals other than fish

The liver fat of sheep and chicken contains no vitamin A_2 (Table I, nos. 4, 5), that of the green frog only small quantities (Table I, no. 6).

These observations confirm and extend the findings of Castle *et al.* [1934], Edisbury *et al.* [1938] and Gillam *et al.* [1938] concerning the absence of the 693 mμ chromogen from mammalian liver fat and seem to indicate that vitamin A_2 is a specific product of the liver metabolism of certain species of freshwater fish.

Exact measurements of the vitamin content of invertebrates have often been impossible because of the minute quantities of chromogenic material isolated or because of the presence of substances causing the formation of a turbidity with SbCl$_3$. Qualitative observations indicate that the intestines of the cephalopod *Loligo vulgaris* contain vitamin A_1 without any trace of vitamin A_2.

Accumulation of vitamin A_2 in the liver of the rat and the frog

The absence of vitamin A_2 from mammalian liver fat might be due to a differential absorption of vitamins A_1 and A_2 from the intestine (cf. the differential absorption of carotene and xanthophyll by the horse intestine observed by

Zechmeister & Tuszon [1935, 2]) or to a more rapid destruction of vitamin A_2 after absorption.

Qualitative feeding experiments have shown that the rat accumulates vitamin A_2 in its liver when receiving orally a concentrate from freshwater fish liver oil in the same way that it accumulates vitamin A_1. The absence of vitamin A_2 from mammalian liver fat can therefore be explained by the absence of vitamin A_2 from the food.

Feeding experiments on frogs, using concentrates of both vitamin A_1 and vitamin A_2, have shown that the amount of vitamin A_1 as well as the ratio vitamin A_2/vitamin A_1 can be increased in the liver.

Increase of the ratio E 695 mμ/E 620 mμ in the liver fat of frogs fed on a vitamin A₂ concentrate

No. of frogs	No. of feedings*	E 695 mμ/E 620 mμ
4	0	0·24
2	4	0·30
2	8	0·30
4	18	0·55

* Two drops of pike-perch liver oil (Table III, no. 2) a day per frog; no other food.

Owing to the slow metabolism of these animals little effect was produced till the feedings were continued for more than a week. Most of the oil fed was found in the intestine of the frogs.

SUMMARY

1. Vitamin A_2 (693 mμ chromogen) gives with antimony trichloride a two-banded spectrum (maxima at 693 and 650 mμ). In unsaponifiable fractions the band at 650 mμ is entirely masked by the 693 mμ band: it can be seen in some natural oils and in partly saponified oils owing to the presence of inhibitors which diminish the intensity of the 693 mμ band. It can be made visible in unsaponifiable fractions by the addition of such inhibitors.

2. The 650 mμ band of vitamin A_2 causes considerable absorption at 620 mμ and therefore only a part of the absorption at 620 mμ observed in vitamin A_2 concentrates is due to the presence of vitamin A_1. Measurements and calculations lead to the conclusion that in concentrates rich in vitamin A_2 (E 695 mμ/ E 620 mμ = 3) nearly half of the absorption at 620 mμ is due to vitamin A_2. Thus E 695 mμ/E 620 mμ of pure vitamin A_2 would lie between 6 and 8.

3. The ultraviolet absorption spectrum of our purest vitamin A_2 concentrates, which are entirely soluble in 77 % ethyl alcohol, shows absorption bands at 345 and 280 mμ.

4. Trichloroacetic acid, dimethyl sulphate, perchloric acid and other reagents give with both the vitamins coloured solutions having about the same bands as those which occur in the Carr and Price reaction.

5. The relative quantities of vitamins A_1 and A_2 (expressed as E 695 mμ/ E 620 mμ) have been measured for a series of livers from freshwater fish from several countries of Europe. It has been found that this ratio is more or less constant for a given species. Thus pike-perch (*Lucioperca lucioperca*), pike (*Esox lucius*), catfish (*Silurus glanis*), and perch (*Perca fluviatilis*) have ratios of 2·0–2·7; trout (*Salmo irideus*), carp (*Cyprinus carpio*), sturgeon (*Acipenser sturio*) and salmon (*Salmo salar*) have low ratios (0·4–0·8). Marine fish (*Gadus merlangus*, *Hippoglossus hippoglossus*, *Ishinagi sterelepis*) and land animals such as sheep

(*Ovis aries*), chicken (*Gallus domesticus*) and frog (*Rana esculenta*) have still lower ratios (0·03–0·27).

6. Intestinal oils of freshwater fish have usually a somewhat lower ratio E 695 mμ/E 620 mμ than the liver oils of the same animals. For the carp, on the contrary, this ratio tends to be higher in the intestine than in the liver.

7. The stomach contents and depot fat around the pyloric caeca of the pike-perch have a ratio E 695 mμ/E 620 mμ = 1·0, whereas the liver and caeca themselves have a ratio of 2·4. A possible explanation for this difference is discussed.

8. Feeding experiments have shown that the rat and the frog can absorb vitamin A$_2$ from the intestine and accumulate it in the liver. Thus the absence of vitamin A$_2$ from the liver of mammals and other land animals can be explained simply by the absence of vitamin A$_2$ from the food.

Vitamin A$_2$ seems to be a specific product of the liver metabolism of certain species of freshwater fish.

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CLXVIII. THE UTILIZATION OF CO₂ BY THE PROPIONIC ACID BACTERIA[1]

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THE utilization of CO_2 by heterotrophic bacteria was first reported by Wood & Werkman [1936, 1] in the case of the fermentation of glycerol by the propionic acid bacteria. Barker [1936] and van Niel [1937] have questioned the evidence for this physiologically important phenomenon. Van Niel states, "Wood and Werkman claim that carbon dioxide is reduced during the fermentation of glycerol by propionic acid bacteria. The published results cannot, however, be considered conclusive, although the data do seem to favor their claim." According to previous concepts of the physiology of heterotrophic, non-photosynthetic bacteria the utilization of CO_2 by the propionic acid bacteria appears unlikely; thus confirmation by other workers is desirable. Neither Barker nor van Niel state the respect in which they consider our data inconclusive. It should be clearly recognized that although theories advanced concerning the mechanism of CO_2 assimilation are necessarily speculative and analyses of the fermentations will show experimental variation, proof of CO_2 utilization does not depend on these points. The proof is simply that a given quantity of CO_2 in the form of $CaCO_3$ was added to a medium which was fermented and analysed. Less CO_2 was accounted for than was originally present in the medium. Moreover, the recovery of the CO_2 from the $CaCO_3$ in an unfermented control medium was quantitative. The fact that the C recovery and oxidation-reduction balance were satisfactory when CO_2 utilization was assumed but were not when the principle of CO_2 utilization was not employed, was considered further evidence substantiating the occurrence of the phenomenon.

The purpose of the present investigation was to obtain additional information concerning the utilization of CO_2 in the dissimilation of glycerol by propionic acid bacteria. It is hoped that these studies will ultimately lead to an elucidation of the mechanism of this utilization.

METHODS

Two procedures have been used involving (1) serial analysis of the fermentation and (2) single analysis at the conclusion of the fermentation. The medium consisted of glycerol 2·5 %, Difco yeast extract 0·4 % and $NaHCO_3$ 1·5 %. Each constituent was sterilized separately. The $NaHCO_3$ solution was made acid to phenolphthalein by saturation with CO_2 before adding it to the medium. Incubation was at 30°. The inocula were suspensions of cells in distilled water, obtained from glycerol yeast extract medium, used in a concentration of 3 % by volume. During incubation the medium was agitated by shaking once each day.

The apparatus used for the serial analysis is shown in Fig. 1. The fermentation was conducted under an atmosphere of CO_2 in a 2 l. Erlenmeyer flask (A) with 1900 ml. medium. The rubber stopper held an inlet (B) for CO_2-saturation of the medium, an outlet (C) leading to the absorption train (D), a syphon (E) for sampling, one connexion leading to a Hempel gas burette (F) and reservoir (G),
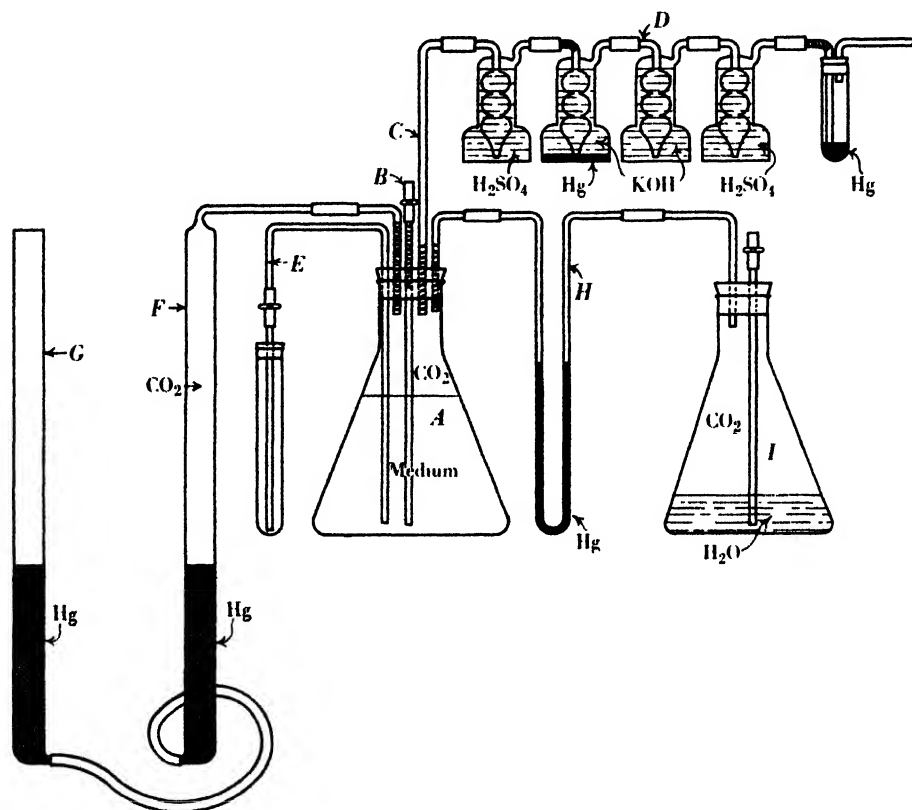
Fig. 1. Fermentation apparatus used in serial experiments.

a second connexion to a U-tube manometer (H) which was in turn connected to a closed flask (I) containing CO_2 and water to keep the gas saturated. The pressure in the fermentation flask was balanced against that of reference flask I by adjustment of the reservoir (G) before reading the volume in the burette and removing a sample. It is convenient to use a reference flask since the apparatus is then not affected by changes in temperature or pressure. There was a liberation of CO_2 during most of the fermentation, in spite of its utilization, owing to the reaction of the $NaHCO_3$ with acids formed by fermentation. This CO_2 was collected in 30 % KOH in two Bowen bulbs in the drying train (D) and determined by weight. Hg was placed in the bottom of the first KOH bulb to cover the tip of the inlet tube to act as a trap between the alkali and CO_2 and prevent the alkali from being pulled back into the gas train. Only after the $NaHCO_3$ had completely reacted was the utilization of gaseous CO_2 apparent. At this time a partial vacuum was created in the fermentation flask and the gas utilized was measured in the burette.

All samples were removed into a known volume of alkali to prevent loss of dissolved CO_2. During removal of the sample, CO_2 was simultaneously introduced into the flask, thus preventing change in the equilibrium of free and dissolved gas. The liquor was thoroughly agitated before sampling to facilitate gaseous equilibrium. The original glycerol and CO_2 in the medium, i.e. dissolved CO_2 plus CO_2 bound as $NaHCO_3$ were determined on a sample removed immediately after the medium was inoculated and saturated with CO_2.

The principles of the apparatus are described in more detail by Dixon [1934] and Wood et al. [1936]. No rubber tubing was used except at glass to glass joints where flexibility was necessary. Burette and manometer were filled with Hg.

In the second procedure when only a single analysis of the fermented medium was made, the apparatus shown in Fig. 2 was used. All connexions were sealed by Hg. The fermentation flask was a 300 ml. Erlenmeyer with a tube fitted by a
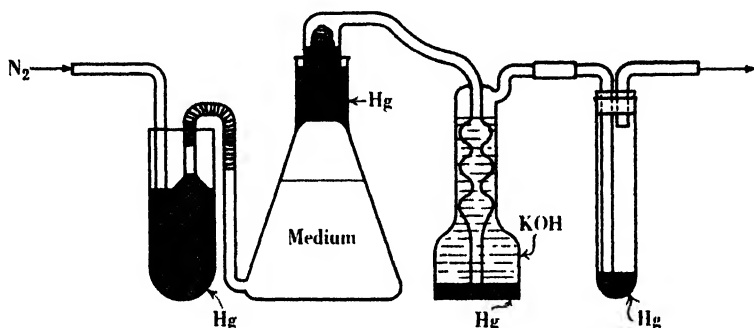
Fig. 2. Fermentation apparatus used in single analysis experiments.

ring seal into the neck. 250 ml. of medium were used in each flask. The incubation time was 30 days. Anaerobiosis was assured by passing O_2- and CO_2-free N_2 through the medium before the addition of the $NaHCO_3$. After this addition the medium was again flushed with N_2 for about 5 min.: longer treatment caused the medium to become alkaline owing to the removal of CO_2. The original CO_2 and glycerol in the $NaHCO_3$ and glycerol solutions were determined before addition to the medium. During the fermentation a slight vacuum was maintained in the apparatus (Fig. 2) thus preventing the development of pressure sufficient to break the Hg seals. After completion of fermentation, the CO_2 was flushed from the medium for 3 hr. with N_2. Because of difficulty of including a drying train in the apparatus (Fig. 2), the CO_2 collected in the KOH was liberated and reabsorbed in a suitable train for determination by wt., adding excess H_2SO_4 (blue to Congo red), boiling the whole under a reflux condenser and aerating. A correction was made for the small amount of CO_2 contained originally in the 30 % KOH.

Other quantitative determinations in both procedures were made as follows. The CO_2 (dissolved CO_2 and $NaHCO_3$) of the fermented medium was determined by wt. on an aliquot part of the sample. The volatile acids and propyl alcohol were determined on the same fraction after removal of the CO_2. The alcohol was concentrated and separated from the volatile acids by distillation of one-half of the volume of the liquor and redistillation after neutralization. The alcohol was determined by oxidation with dichromate and analysis of the resulting acids (unpublished method). The volatile acids were obtained from the acidified

combined residues of the two alcohol distillations by steam distillation and were determined by the partition method of Osburn *et al.* [1933].

Succinic acid was extracted with ether from an aliquot part of the sample acidified with H$_2$SO$_4$ and determined gravimetrically as the Ag salt after removal of the volatile acids from the extract by steam distillation. The titratable acid in the distilled extract was equivalent to the succinic acid obtained in the Ag salt, also the Ag content of the salt was in agreement with the theoretical value. A separate aliquot part of the unheated liquor was extracted because apparently the glycerol and succinic acid react during the distillation. Previous results reported by Wood & Werkman [1936, 1] may show some error in the succinic acid since the determinations were made on extracts obtained from the residues of steam distillations which were taken up in Na$_2$SO$_4$. Recovery of succinic acid by this method is low when glycerol is present but complete when absent.

The unfermented glycerol was determined in an aliquot part of the medium by oxidation with periodic acid (unpublished method). This oxidation is specific for polyhydroxy and related compounds. The medium in the absence of glycerol gives a negligible determination and none of the products reacts. Pyruvic and lactic acids were not found at any time during the fermentation and there was no reduction of Fehling's solution by the fermentation liquor after hydrolysis with acid. Total C determinations were made by the method of Osburn & Werkman [1932] on the acidified medium from which residual CO$_2$ was removed. All analyses were made on the unfermented medium and the small blank values thus obtained were subtracted from the determinations made on the fermented medium.

EXPERIMENTAL RESULTS

Results of serial analysis of the fermentation by *Propionibacterium pentosaceum* are presented in Tables I and II. The data in Table I are calculated on the basis of 1 l. medium for the period t_0 to the time of removal of the respective samples. The results in Table II are calculated on the basis of the fermentation occurring during the time between samplings and per 100 mM. glycerol fermented. Data obtained by single analyses of the fermentation are given in Table III.

The accuracy of the data can be judged from the C recovery and oxidation-reduction balance; the former (Table I) is low during the early period but increases as the fermentation proceeds. On the basis of the glycerol fermented and CO$_2$ utilized the C unaccounted for is substantially constant after the 9th day as shown in Table I. The maximum cell development is reached on the 7th or 8th day and remains quite constant thereafter. This simultaneous occurrence

Table I. *Serial analysis of the dissimilation of glycerol by* Propionibacterium pentosaceum (*49 W*)

Time days	Glycerol fermented per l. mM.	CO$_2$ utilized per l. mM.	Products per l.				C unaccounted for per l. mM.	C recovery %	Redox-index O/R
			Succinic acid mM.	Propionic acid mM.	Acetic acid mM.	Propyl alcohol mM.			
4	36·55	5·34	4·98	24·9	1·49	1 42	13·0	88·7	1·04
6	66·26	10·53	10·20	43·8	2·46	3·84	20·5	90·2	1·01
9	114·40	23·90	23·10	73·8	3·56	6·61	26·4	92·8	0·97
12·5	160·90	41·80	41·00	101·9	4·31	6·64	26·2	95·0	0·98
19·5	191·60	53·60	51·60	120·1	4·16	7·66	30·1	95·2	0·97
28·5	222·30	70·50	68·60	136·7	8·18	4·66	22·1	97·0	1·00
46·5	275·20	103·80	99·80	159·0	5·94	4·69	26·9	97·1	0·99

Table II. *Serial analysis of the dissimilation of glycerol by* Propionibacterium
pentosaceum (*49 W*)

Interval days	Glycerol fermented per l. mM.	CO_2 utilized per 100 mM. glycerol mM.	Products per 100 mM. glycerol				C recovery %	Redox-index O/R
			Succinic acid mM.	Propionic acid mM.	Acetic acid mM.	Propyl alcohol mM.		
0–4	36·55	14·6	13·6	68·3	4·08	3·87	88·7	1·04
4–6	29·71	17·5	17·6	63·6	3·26	8·15	92·0	0·96
6–9	48·10	27·8	26·7	62·2	2·29	5·76	96·2	0·94
9–12·5	46·50	38·3	38·6	60·5	1·61	0·06	100·2	1·01
12·5–19·5	30·60	38·5	34·7	59·4	− 0·49	3·34	96·3	0·92
19·5–28·5	30·70	55·5	55·2	54·1	13·09	− 9·77	106·4	1·12
28·5–46·5	52·90	62·9	59·0	42·1	− 4·23	0·06	97·7	0·95

Table III. *Dissimilation of glycerol by the propionic acid bacteria.**

Culture*	Glycerol fermented per l. mM.	CO_2 utilized per 100 mM. glycerol mM.	Products per 100 mM. glycerol				C recovery %	Redox-index O/R
			Succinic acid mM.	Propionic acid mM.	Acetic acid mM.	Propyl alcohol mM.		
11 W	214·3	12·6	16·6	80·00	3·97	1·42	101·9	1·06
34 W	212·5	10·4	15·0	76·40	3·30	3·32	98·4	1·08
35 W	212·3	13·5	16·9	77·10	2·74	2·77	99·7	1·04
49 W	211·5	17·2	20·2	76·60	1·38	1·95	100·7	1·03
52 W	206·7	21·1	25·1	74·19	2·08	0·19	102·0	1·07

* Culture numbers correspond to the following species: 11 W, *P. petersonii*; 34 W, *P. arabinosum*; 35 W, *P. zeae*; 49 W, *P. pentosaceum* and 52 W, *P. shermanii*.

of cell development and low C recovery suggests that for the most part the C unaccounted for occurs as cell substance. The redox-index is satisfactory in almost every case in Table I. There is more variation in the balances shown in Table II than in those of Table I which is the result of the small values involved in the calculations of the data of Table II. The largest discrepancy is in the period between 19·5 and 28·5 days. When the data are considered as a whole and the C in cell material is taken into account the results are acceptable. The redox-indexes in Table III show some variations from 1·0; however, a small error in the analysis can cause such discrepancies.

A large utilization of CO_2, which started with initial fermentation and increased thereafter, is shown by Tables I and II. Since Barker and van Niel have previously questioned the validity of similar evidence of CO_2 utilization it seemed advisable to obtain further proof. The organic C in the medium should increase by an amount equivalent to the CO_2 utilized, since there is no loss of C as gaseous products. This relationship has been established by total C determinations on the medium before and after fermentation. The C determinations were made on the medium after bound CO_2 (NaHCO$_3$ and dissolved CO_2) had been removed. Aliquot parts of the original sample (unfermented) and the final sample (fermented for 46·5 days) equivalent to exactly 3 ml. undiluted medium were used in the determinations. The $K_2S_2O_8$ oxidation gave 0·1235 g. CO_2 per 3 ml. in the unfermented medium and 0·1355 g. CO_2 in the fermented medium, an increase of 0·0120 g. Experimentally 103·8 mM. of CO_2 per l. were utilized (Table 1, 46·5 days) or 0·0137 g. CO_2 per 3 ml. The fact that the CO_2 utilized, as determined experimentally, agrees closely with the increase in organic C, substantiates the utilization of CO_2 and is strong evidence for its conversion into organic compounds.

Further evidence of CO$_2$ utilization was shown by the formation of a partial vacuum in the fermentation flask in the interval between 12·5 and 19·5 days. During this time the NaHCO$_3$ in the medium was entirely decomposed and the utilization was no longer masked by evolution of CO$_2$: 123 ml. CO$_2$ at N.T.P. were utilized per 1. medium.[1]

Propyl alcohol was a product of the fermentation of glycerol in the present investigation. The authors have not found previous mention of the formation of this compound by propionic acid bacteria. Its isolation and identification will be described elsewhere. The alcohol obtained in the present investigation yielded after oxidation with dichromate a mixture of propionic and acetic acid in exactly the same proportion as from authentic propyl alcohol.

DISCUSSION

The experimental results show clearly that substantial quantities of CO$_2$ are utilized by the propionic acid bacteria in the dissimilation of glycerol. It is of primary interest to obtain an insight into the mechanism of CO$_2$ utilization and to determine whether the phenomenon occurs in other biological processes.

The results in Tables I, II and III bring out one point which may give an indication of the mechanism of CO$_2$ utilization. The mM. of CO$_2$ utilized and succinic acid formed show a close relationship in all the fermentations. This approximate equivalence of the succinic acid and CO$_2$ suggests that the former may originate by union of CO$_2$ with some 3-C compound formed from the glycerol. In this connexion the possibility must be considered of the formation of CO$_2$ from glycerol and its utilization together with that from the NaHCO$_3$. Since a 2-C compound (acetic acid) is formed from a 3-C substrate (glycerol), it is only reasonable to expect the formation of an equivalent concentration of a 1-C compound (CO$_2$). In this case the total CO$_2$ utilized will be equal to the sum of the CO$_2$ obtained from the NaHCO$_3$ (3rd column, Tables I-III) and that originating from glycerol with the acetic acid. Comparison of this sum (Table III) with the values obtained for succinic acid (16·6–16·6, 13·7–15·0, 16·3–16·9, 18·6–20·2, 23·2–25·1) discloses that the agreement is within limits of experimental error. The results shown in Table I[2] are not in complete agreement with this explanation. If to the CO$_2$ utilized from the NaHCO$_3$ a quantity is added equivalent to the observed acetic acid, then there is obtained a significant excess of total CO$_2$ over the succinic acid. It is probable that the results in Table III are more reliable than those in Table I inasmuch as the latter were obtained by a more complex procedure. However, the redox-indexes are more satisfactory in Table I. It is not deemed advisable to propose reactions to explain these small variations until additional evidence is available. Possibly part of the succinic acid was broken down to acetic acid without evolution of CO$_2$. There is, however, a general correlation of CO$_2$ utilization with succinic acid formation suggesting a synthesis involving CO$_2$ and succinic acid. Results reported in a previous paper [Wood & Werkman, 1936, 1] do not show an equimolar formation of CO$_2$ and succinic acid. However, as previously indicated, the method used in the determination of succinic acid was not accurate in all cases. For this reason present experiments are a better criterion of the CO$_2$-succinic acid relationship.

[1] The pH of the medium was 5·5 on the 19·5 day. Sufficient alkali was therefore added to buffer the medium throughout the remainder of the fermentation and it was again saturated with CO$_2$. A sample was then removed for determination of bound CO$_2$.

[2] Table II is not considered here because the method of calculation multiplies the experimental error.

During the preparation of this manuscript the publication of Elsden [1938] appeared, showing that the rate of succinic acid formation by washed suspensions of *Bacterium coli commune* from sodium pyruvate, glucose and galactose is a function of the concentration of CO_2 in the medium. No direct evidence was obtained for fixation of CO_2. It was suggested that possibly CO_2 is involved in the synthesis of succinic acid but the facts were too few to permit theorizing on the role of CO_2 in succinic acid formation. In the view of present results which show that succinic acid, a 4-C compound, is formed from the 3-C compound glycerol in amounts approximately equivalent to the CO_2 utilized, the definite possibility that succinic acid may be formed by synthesis from 3-C and 1-C compounds is evident. In the light of this suggestion an explanation is available for the results obtained by Virtanen [1925] and Virtanen & Karström [1931] who found succinic and acetic acids in equimolar quantities as the only products from glucose using preparations of propionic acid bacteria in the presence of toluene. These results could not be explained logically on the basis of a 3-C cleavage, i.e.

$$\text{one } 6\text{-C} = \text{two } 3\text{-C} = \text{two } 2\text{-C} + \text{two } 1\text{-C; and two } 2\text{-C} = \text{one } 4\text{-C,}$$

since CO_2 utilization was not considered at that time. The formation of succinic and acetic acids by a 4- and 2-C cleavage was therefore proposed to account for the formation of succinic and acetic acids unaccompanied by a 1-C compound. It is quite evident that Virtanen & Karström's results are precisely those to be expected in view of the present proposal, i.e. that one hexose yields two 3-C compounds; one is converted to acetic acid and CO_2 and the other unites with CO_2 or some derivative of CO_2 to give succinic acid.[1] Other investigators, Virtanen [1928], Scheffer [1928], Braak [1928] and Kluyver [1931], have applied the idea of 4- and 2-C cleavage to bacterial fermentations primarily because it offered an explanation for the shortage of 1-C compounds.

It is of interest that Butkewitsch & Gaewskaya [1935] and Wells *et al.* [1936] claim that the formation of citric acid from sugar by moulds cannot proceed by synthesis from 2-C compounds which originate from 3-C compounds. This view is maintained since they obtained more than the theoretical yield of citric acid, i.e. if 1-C compounds are not utilized. Gudlet & Makarowa [1935] proposed a 4-and 2-C cleavage of glucose with a subsequent synthesis as a possible mechanism in the formation of citric acid. Previously Bernhauer & Slanina [1934] showed that fungi can synthesize oxalic acid from formic acid and pointed out that there is no arbitrary limit to the quantity of citric acid which may be formed from glucose if synthesis occurs. The merits of the different schemes of citric acid formation are not within the scope of this investigation but it should be emphasized that it is not possible to limit the quantity of citric acid which can be formed from glucose since CO_2 utilization may also occur in mould metabolism. Woods [1936] has shown that *Escherichia coli* can reduce CO_2 to formic acid. This conversion does not involve a C to C linkage but does show that CO_2 can be activated by *Esch. coli*. Wieringa [1936] claims to have isolated an anaerobic spore-forming bacterium which almost quantitatively forms acetic acid from CO_2 and H_2. The methods and data necessary to support the claim are not presented in his brief report but his discovery may be another example of the chemosynthetic activity of heterotrophic bacteria. If the propionic acid bacteria and the anaerobic sporulating bacterium of Wieringa are oligocarbophilous there

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[1] There is one inconsistency in Virtanen' & Karström's results. They obtained an oxidized product, succinic acid, with no corresponding reduced product, acetic acid being neither oxidized nor reduced.

is no reason *a priori* to assume that moulds and other tissues are not similar. There is no direct proof of the occurrence of a 4- and 2-C cleavage in biological reactions and many of the past concepts of fungous and bacterial metabolism may have to be re-evaluated on the basis of CO_2 utilization. Krebs & Johnson [1937] have recently shown that citric acid is synthesized by avian tissue from oxaloacetic acid and some unknown compound. It is possible that this synthesis involves utilization of CO_2.

Previously Wood & Werkman [1936, 1, 2] and Wood *et al.* [1937] have shown that certain glucose fermentations by propionic acid bacteria yield relatively large quantities of succinic acid and CO_2 and a small quantity of acetic acid. These results are readily adaptable to a scheme of fermentation involving the formation of succinic acid from acetic acid. On the contrary, if the succinic acid is assumed to be formed by union of CO_2 (or some other 1-C compound) and a 3-C compound, it is difficult to explain the origin of the large quantities of CO_2 in comparison with the small amount of acetic acid. Two mechanisms have previously been proposed for the formation of CO_2; one by the breakdown of pyruvic acid to acetic acid and CO_2; the other by decarboxylation of succinic acid to propionic acid and CO_2. If these reactions were the only sources of CO_2, and succinic acid was formed by union of CO_2 and a 3-C compound, the CO_2 would of necessity be less than the acetic acid provided that the latter was not broken down. If succinic acid formation occurs in glucose dissimilation solely by CO_2 synthesis it is probable that there is a mechanism of CO_2 formation which has not been considered as yet. Stone *et al.* [1936] and Wood *et al.* [1937] have shown that acetic acid is activated and dissimilated by propionic acid bacteria. In fact the results in Table I indicate there may have been some breakdown of acetic acid between 28·5 and 46·5 days. Aerobically, resting cells of propionic acid bacteria can attack acetic acid slowly (unpublished results). At present it seems probable that there may be two mechanisms of succinic acid formation in glucose dissimilation: one by CO_2 synthesis and another by synthesis involving acetic acid [Wood & Werkman, 1936, 2].

Although the present observations showing the equivalence of CO_2 and succinic acid indicate that the latter is formed predominantly by CO_2 synthesis in glycerol fermentation, other interpretations can be given. The formation of the oxidized product, succinic acid, must be accompanied by a simultaneous reduction. Since the utilization of CO_2 involves reduction, the relationship of CO_2 and succinic acid may arise in the establishment of the oxido-reduction balance.

The close connexion of CO_2 to succinic acid formation has also been shown by fermentation of glycerol under N_2 with phosphate as a buffer. Numerous experiments have been conducted but in no case have substantial quantities of succinic acid been found as a product under these conditions, i.e. in the absence of CO_2. Propionic, acetic, pyruvic and lactic acids and propyl alcohol are formed with no gas. These experiments indicate that CO_2 is necessary for the formation of succinic acid and are in complete agreement with the investigations of Elsden [1938].

The relative change in the course of the fermentation with time as shown in Table II is of interest. On the basis of the glycerol fermented, the CO_2 utilized and succinic acid formed during each interval progressively increased whereas the propionic acid decreased. Comparing the first with the final analysis, the mM. CO_2 utilized increased from 14·6 to 62·9 and the succinic acid from 13·6 to 59·2 mM. while the propionic acid decreased from 68·3 to 42·1, per 100 mM. glycerol. The cause of this change is not clear but it illustrates how large the utilization of CO_2 can be and also the relative changes in the other products.

The CO_2 amounted to 17·3 % of the total C converted, on the basis of the glycerol fermented and CO_2 utilized between 28·5 and 46·5 days.

The complete mechanism of the fermentation of glycerol by propionic acid bacteria is not clear. The role of phosphate esters in this fermentation has not been investigated extensively. Werkman et al. [1937] have shown that the fermentations of glycerophosphoric and phosphoglyceric acids are inhibited by 0·02M NaF. On the contrary, the fermentation of glycerol with proliferating cells proceeds in this concentration of fluoride (unpublished results). Apparently glycerophosphoric acid and phosphoglyceric acid are not necessary intermediary compounds. Wiggert & Werkman [1938] have recently shown that propionic acid bacteria grown in the presence of fluoride do not have an enzyme system for the breakdown of phosphoglyceric acid, whereas in the absence of fluoride this enzyme system develops. Perhaps this also occurs in the fermentation of glycerol, in which case, in the absence of fluoride, phosphoglyceric acid and glycerophosphoric acid may be intermediates. Wood & Werkman [1938] have shown that glycerophosphoric acid is fermented to the same final products as glycerol and, on this basis, they could not exclude the ester as an intermediate of glycerol fermentation. Whether or not CO_2 uptake is connected with phosphorylation is not known at present. Pyruvic acid probably occurs as an intermediate since it may be isolated from glycerol fermentations by sulphite fixation [Wood et al. 1937]. This compound may be the point of entrance for CO_2. Apparently propyl alcohol can be dissimilated by propionic acid bacteria. There was a decrease in the actual amount of propyl alcohol at the conclusion of the fermentation (Table I). Propyl alcohol is probably formed by reduction of propaldehyde which has been isolated from glycerol fermentation by fixation with sulphite and dimedon [Wood & Werkman, 1934].

Recently Fromageot & Bost [1938] have reported that the succinic acid which they obtained in propionic fermentation probably originated from compounds of the yeast extract. The CO_2 was not determined in their experiments but was calculated from arbitrary equations. Wood & Werkman [1936, 2] have shown that similar equations are not completely adaptable to the propionic acid fermentation of glucose. The excessive C recoveries (based on calculated CO_2 values) obtained by Fromageot & Bost are not a strictly reliable indication that products are being formed from the yeast extract, inasmuch as no consideration was given to the possibility of CO_2 utilization. It is probable that small quantities of different products are formed from the yeast extract; however, there is no evidence that these products are confined to succinic acid. In our experiments in which Difco yeast extract (0·4 %) has been used as the source of N there has been no evidence that the quantities of products formed from the yeast extract are significant when compared with a fermentation of 2·5 % substrate.

SUMMARY

Previous experiments showing utilization of CO_2 by propionic acid bacteria fermenting glycerol have been confirmed and extended. The C of the utilized CO_2 has been found in the products, which are succinic, propionic and acetic acids and propyl alcohol.

Total C determinations before and after fermentation show that there is an increase in the total organic C of the medium equivalent to the decrease in inorganic C (CO_2).

The uptake of gaseous CO_2 was sufficient to create a partial vacuum in the apparatus.

The succinic acid formed and CO_2 utilized are approximately equimolar. It is suggested that the formation of succinic acid is by synthesis from a 3-C compound through addition of CO_2.

In the absence of CO_2 little or no succinic acid is formed.

The relative proportions of the products varied with time and the CO_2 utilized. The succinic acid formed increased while the propionic acid decreased.

Utilization of CO_2 by heterotrophic bacteria is considered in connexion with other reactions proposed for the formation of succinic and citric acids.

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CLXIX. THE ACTIVATION OF FEMALE SEX HORMONES

III. MONO-ESTERS OF α-OESTRADIOL

By K. MIESCHER, C. SCHOLZ and E. TSCHOPP

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Two series of mono-esters are derived from α-oestradiol according to whether the acid radical is in the 3- or 17-position.

Up to the present only one mono-ester has been thoroughly investigated, viz. oestradiol-3-benzoate prepared by Schwenk & Hildebrandt [1933]. According to Kaufmann [1933; see also Schoeller *et al.* 1935] it has a protracted effect in comparison with the free follicular hormone. In the previous paper of this series [Miescher *et al.* 1938, 2] we showed that as regards duration of effect and output of activity many di-esters of oestradiol are much better than the 3-benzoate. The same applies also to the series of the 3- and 17-mono-esters. The investigation of the esters was made by determining their threshold value and duration of effect in the oestrus test as well as their action in the uterus growth test using rats in the same manner as described previously. Again the esters were injected subcutaneously in oily solution. 1275 animals were used for these experiments.

The mono-esters of α-oestradiol investigated by us are shown in Table I.

Table I

Oestradiol mono-esters	M.P.* °C.	Oestrus test (rat)	
		Threshold value S_r rat oestradiol $= 0.4\gamma$	Threshold value S_o based on oestradiol $= 0.1\gamma$
3-Acetate	136.5–137.5	0.4	0.1
3-Propionate	125.0–126.0	0.4	0.1
3-n-Butyrate	98.0– 99.0	0.4	0.1
3-n-Valerate	Approx. 58.0– 60.0	0.4	0.1
3-n-Hexanoate	Approx. 46.0– 51.0	0.4	0.1
3-n-Octanoate	Approx. 48.0– 53.0	0.6	0.2
3-n-Decanoate	59.0– 60.0	2.0	0.5
3-Palmitate	70.0– 71.0	7.0	1.8
3-Stearate	78.0– 78.5	7.0	1.8
3-Benzoate	193.0–194.0	0.75	0.2
17-Acetate	215.0–216.5	0.5	0.1
17-Propionate	199.0–200.0	0.5	0.1
17-n-Butyrate	166.5–167.0	0.5	0.1
17-isoButyrate	183.0–183.5	0.5	0.1
17-n-Valerate	144.0–145.0	0.5	0.1
17-n-Hexanoate	128.5–129.0	0.7	0.2
17-n-Octanoate	117.5–118.0	0.8	0.2
17-n-Decanoate	112.0–112.5	3.5	0.9
17-Benzoate	93.0– 94.0	3.0	0.8
17-Methylcarbonate	216.5–218.0	0.6	0.2
17-Ethylcarbonate	171.0–172.0	0.6	0.2
3:17-Diethylcarbonate	137.5–138.0	1.5	0.4

* The melting-points are taken with a short thermometer.

Apart from the 3-benzoate only the two mono-acetates have been mentioned in the literature. The preparation of a series of mono-esters has already been reported by us elsewhere [Miescher & Scholz, 1937, 1, 2]. A description of some further mono-esters is appended.

Oestradiol-3-n-valerate. 630 mg. oestrone-*n*-valerate were dissolved in 95 ml. ethyl acetate (containing no alcohol) and mixed with 350 mg. platinum oxide (Adams). The whole was shaken at room temperature under a pressure of hydrogen of 4·3 atm. for 20 hr. After filtering off the platinum and evaporation of the solvent under diminished pressure the remaining oil was purified for analysis by two distillations at 0·0004 mm. (bath temperature 180–190°). (Found: C, 77·61; H, 9·01%. $C_{23}H_{32}O_3$ requires C, 77·47; H, 9·06%.) The oil can be crystallized from alcohol-water but the crystals contain solvent. M.P. about 58–60 .

Oestradiol-3-n-hexanoate. A solution of 428 mg. oestrone-*n*-hexanoate in 65 ml. alcohol-free ethyl acetate was mixed with 220 mg. platinum oxide and reduced as above. The working up was the same. After distilling 3 times at 0·0008 mm. (bath temperature 180–190°) a colourless oil was obtained which could be crystallized from alcohol-water; however, the crystals contain solvent. The M.P. is about 46–51°. (For the oil, found: C, 77·81; H, 9·20%. $C_{24}H_{34}O_3$ requires C, 77·78; H, 9·26%.)

Oestradiol-3-n-octanoate. In the same manner oestrone-*n*-octanoate, M.P. 69·8–70·2′, was reduced by means of platinum oxide in ethyl acetate and an oily residue obtained which was distilled 3 times at 0·0004 mm. (bath temperature 180). The oil was dissolved in alcohol-water and after standing, crystals were formed, containing solvent. M.P. about 48–53°. (For the oil, found: C, 78·45; H, 9·46%. $C_{26}H_{38}O_3$ requires C, 78·33; H, 9·62%.)

Oestradiol-3-n-decanoate. The oestrone-*n*-decanoate was reduced in the manner described above. After filtering and evaporation of the ethyl acetate under diminished pressure an oil remained which after recrystallizing several times from methanol gave fine needles with constant M.P. 59–60°. (Found: C, 77·05; H, 9·97%. $C_{28}H_{42}O_3$, 0·5 H_2O requires C, 77·18; H, 9·95%.)

Oestradiol-17-n-hexanoate. 960 mg. oestradiol-di-*n*-hexanoate (an oil) were mixed with 60 ml. of 0·5% K_2CO_3 in 95% methyl alcohol and stirred for about 3 hr. at room temperature during which time solution occurred. The solution was then slightly acidified and by slowly adding water crystals were precipitated. After filtering off and thoroughly washing first with water and then with diluted Na_2CO_3 and again with water, almost pure oestradiol-17-*n*-hexanoate was obtained and recrystallized several times from methanol-water. M.P. 128·5–129 . (Found: C, 77·67; H, 9·29%. $C_{24}H_{34}O_3$ requires C, 77·78; H, 9·26%.)

Oestradiol-17-n-octanoate. Partial saponification of oestradiol-di-*n*-octanoate (an oil) with 0·5% K_2CO_3 in 95% methyl alcohol was carried out as above. The working up was the same. After purifying by recrystallization several times from aqueous methanol the crystals had M.P. 117·5–118°. (Found: C, 78·37; H, 9·85%. $C_{26}H_{38}O_3$ requires C, 78·33; H, 9·62%.)

I. *Assay in the oestrus test*

3-Mono-esters. As will be seen from Table I the threshold value in the oestrus test, determined on the rat, is the same from the 3-acetate up to the 3-hexanoate, and is the same as that of free oestradiol ($S_r = 0·4\gamma$). From the 3-octanoate it increases but does not exceed 7γ even with the palmitate and stearate. As regards duration of oestrus (see Fig. 1) with doses of 50γ only a slight increase of the duration of effect is to be seen up to the hexanoate (from 4 to 8 days). With the 3-octanoate the effect increases suddenly up to 52 days and attains with the 3-stearate a maximum of 80 days[1]. On the other hand, the duration of effect with similar doses of the 3-benzoate is only 15 days. With the lower esters up to the 3-octanoate, oestrus appears on the 4th day, that is, one day later than with oestradiol. With the higher esters oestrus appears on the 5th day.

17-Mono-esters. In comparison with the 3-mono-esters the threshold value of this series is slightly increased. The most pronounced difference is observed with

[1] According to a private communication from Prof. Schoeller, Berlin, the 3-palmitate in a previous experiment on a monkey also showed a long-lasting effect.

the 17-benzoate (3γ) compared with the 3-benzoate (0.75γ). As regards the duration of oestrus (Fig. 2) the 17-mono-esters exceed the corresponding 3-mono-esters considerably. Using 50γ the 17-acetate and 17-propionate show the same duration of effect as the 3-benzoate (15 days). With the higher esters it increases
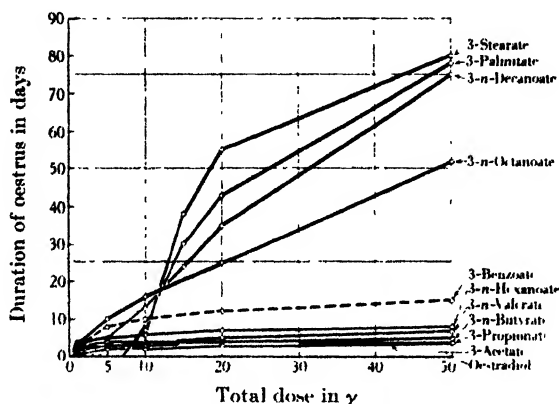
Fig. 1. The effects of aliphatic 3-mono-esters of oestradiol in comparison with oestradiol and its 3-benzoate on castrated rats in the oestrus test, using various doses.

rapidly. The 17-octanoate is of particular interest as, especially with comparatively small doses, it exhibits the most intense action of all esters hitherto investigated. With 5γ the oestrus lasts 30 days, with 20γ 60 days and with 50γ 86 days. With the higher esters (decanoate) the effect appears to diminish. The duration of effect of 17-benzoate (60 days) is 4 times that of the 3-benzoate (15 days). With the 17-mono-esters onset of oestrus is rather more rapid than with the 3-mono-esters. All the 17-mono-esters investigated show their full effect on the 4th day.
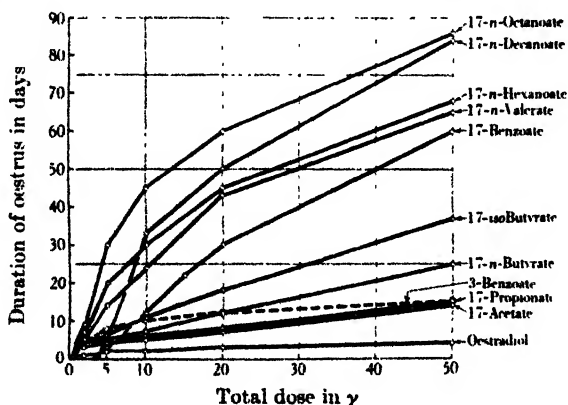
Fig. 2. The effects of 17-mono-esters of oestradiol in comparison with oestradiol and its 3-benzoate on castrated rats in the oestrus test, using various doses.

In Fig. 3 the threshold values (S_r) and duration of oestrus (T_{oe}) of the 3- and 17-mono-esters (with doses of 50γ) are given for comparison. The superiority of the 17-esters over the corresponding 3-esters as regards duration of oestrus is clear. A comparison of this figure with Fig. 2 in the previous paper [1938, 2] is

very instructive. It will be seen that with doses of 50γ none of the 3- and 17-mono-esters studied is equal to the most active di-esters as regards duration of effect.

In addition to these 17-mono-esters we examined also two 17-monocarbonic esters as well as one 3:17-di-ester of oestradiol using a dose of 50γ. The duration of oestrus is with the 17-methylcarbonate 14 days, with the 17-ethylcarbonate 25 days and with the diethylcarbonate of oestradiol 40 days.
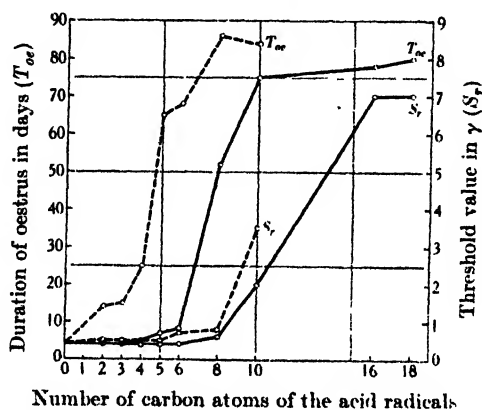
Fig. 3. Comparison of the aliphatic oestradiol -3-mono-esters (· ———–) and -17-mono-esters (– – – –) as regards the threshold value (S_r) and duration of effect (T_{oe}) in the oestrus test.

II. *Uterus growth test*

3-Mono-esters. As in the oestrus test the lower aliphatic 3-mono-esters show also in the uterus growth test (Fig. 4) first of all only a slight increase of effect
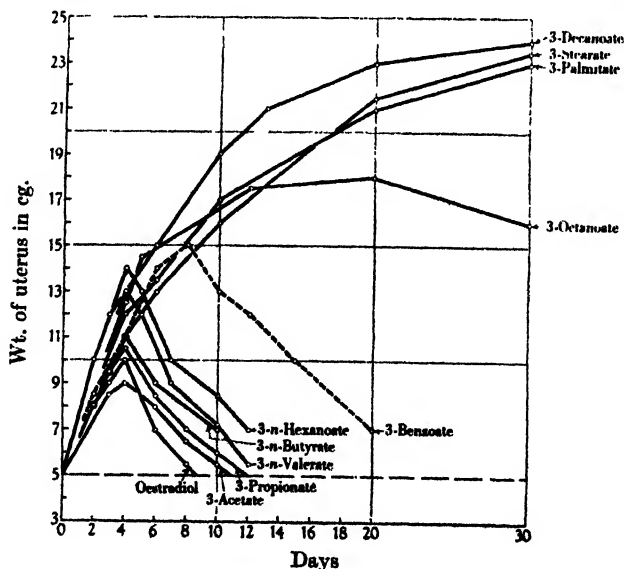
Fig. 4. The effects of aliphatic 3-mono-esters of oestradiol in comparison with oestradiol and its 3-benzoate on the weight of the uterus of young rats after subcutaneous injection of a total dose of 50γ of hormone given in equal parts on two consecutive days.

with the increasing length of the acid radical. On the other hand, the 3-octanoate, and particularly the higher esters, exhibit more pronounced effects. As regards effect the 3-benzoate is between the 3-hexanoate and 3-octanoate. The lower esters up to the 3-hexanoate attain their maximum effect on the 4th day. With the 3-octanoate it is delayed to the 20th day, the maximum effect of the higher esters appearing still later.

17-Mono-esters. The intensive action of the esters substituted in the 17-position is very obvious (Fig. 5). Already with the 17-propionate the intensity of effect obtained with the 3-benzoate is reached, yet as regards duration the former is superior (more than 30 days against 20 days). From the 17-*n*-valerate onwards all higher esters show particularly pronounced effects; even after 30 days the action is still maintained. In comparison with the 3-mono-esters the maximum effect of the corresponding lower 17-mono-esters is delayed. Generally it may be said that the higher the activity of the ester the later the maximum is reached.
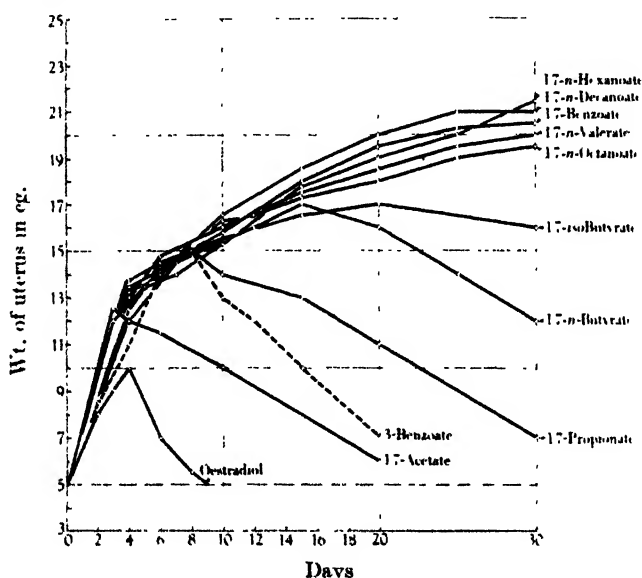
Fig. 5. The effects of 17-mono-esters of oestradiol in comparison with oestradiol and its 3-benzoate on the weight of the uterus of young rats after subcutaneous injection of a total dose of 50γ of hormone given in equal parts on two consecutive days.

The already mentioned carbonates were examined also as regards their effects in the uterus growth test. The 17-monoethylcarbonate with an output of activity of 204 cg.-days was twice as active as the 17-monomethylcarbonate (approx. 100 cg.-days). The diethylcarbonate of oestradiol shows an action similar to that of the oestradiol-17-isobutyrate.

III. *Comparison of the effects on the uteri of castrated and uncastrated animals*

Up to the present our results have been mainly obtained with young uncastrated rats. Experiments with oestradiol-17-octanoate and -dipropionate on a great number of castrated rats demonstrate that during the comparable interval of the first 30 days the corresponding values obtained agree in an astonishing way (see Fig. 6), especially considering the different weights of the

two kinds of animals. However, it should be taken into account that the average weight of the uteri of the castrated untreated rats which we used was approximately the same as that of the young rats. In any case the use of young uncastrated rats for the testing work during the first 30 days is quite justified.
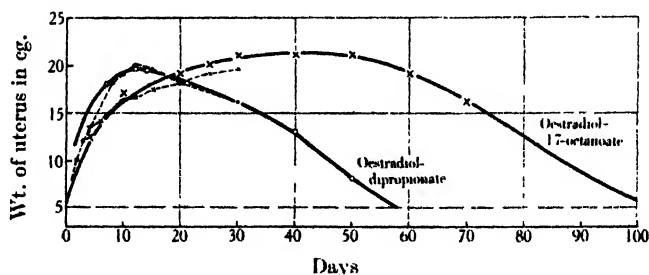
Fig. 6. Comparison of the effects of oestradiol-dipropionate and oestradiol-17-octanoate on the weight of the uteri of young uncastrated rats (– – – –) and of castrated rats (– ·) after subcutaneous injection of a total dose of 50γ hormone given in equal parts on two consecutive days.

IV. *Characterization of the effect of the investigated compounds*

As in the previous publications the effects of the compounds dealt with in this paper have been characterized systematically, in so far as complete data are available, according to their output of activity, A, onset of the maximal effect, T, etc. With all higher esters the output of activity is above 300 cg.-days and attains quite considerable values.

Table II

	Absolute values					Relative values Based on $c_o = 5$ cg.		Based on oestradiol $= 1$
Substance	A cg.-days	D_{max} cg.	t_{max}	T days	D_m cg.	d_{max} %	d_m %	c
Oestradiol	20·3	5·0	4	9	2·3	100	46	1·0
-3-acetate	22·6	4·0	4	11	2·0	80	40	1·1
-3-propionate	30·0	5·5	4	12	2·5	110	50	1·5
-3-n-butyrate	37·2	6·0	4	13*	2·9	120	58	1·8
-3-n-valerate	46·0	8·0	4	13	3·5	160	70	2·3
-3-n-hexanoate	59·6	9·0	4	14*	4·2	180	84	2·9
-3-benzoate	118·8	10·0	8	24*	4·9	200	99	5·9
-17-acetate	86·0	7·5	3	22	3·9	150	78	4·2
-17-propionate	194·0	10·0	8	34*	5·7	200	114	9·6
-17-n-butyrate	327·0	12·0	15	46*	7·1	240	142	16·1
-17-n-octanoate	1100·0	16·0	30	100*	11·0	320	220	54·0
-17-methylcarbonate	100·0	8·0	7	24*	4·2	160	84	4·9
-17-ethylcarbonate	204·0	9·0	7	37*	5·5	180	110	10·1

* Estimated.

V. *Histological observations after administration of oestrone and oestradiol esters*

It is known that the injection of follicular hormone into female rodents causes characteristic modifications in the structure of the mucous membrane of the uterus and vagina.

The modification produced by esters of oestrone and oestradiol depends on the dose given and on the intensity and duration of effect of the derivatives concerned. After giving rats 50γ (administered subcutaneously in two equal doses on

consecutive days) of one of the esters investigated by us, the histological picture of the uteri shows a pronounced hypertrophy of all tissue layers so long as the typical cornified non-nucleated epithelial cells are present in the vaginal smear. Macroscopic examination shows that this organ is enlarged considerably and during the first days of the experiment is filled with liquid and mucus. With the shorter-acting esters the liquid diminishes from about the 6th to the 15th day and from the 15th to the 20th day in the case of the longer-acting esters.

When the effect is mild, the epithelium of the mucous membrane consists first of all of high cellular elements in a single layer. With esters exhibiting a more intense and more prolonged effect other modifications occur which are to be seen particularly in the epithelium. Whereas in the musculature and the mucous membrane a large number of eosinophil leucocytes appear, characteristic changes are perceptible on the epithelium. In the place where single-layered cylindrical epithelium was to be expected, with esters having a prolonged effect several strata of partly keratinized cells of very high cylindrical epithelium ("vertical keratinization" according to Freud [1937]) are found. The multiplicity of layers can be very pronounced at certain places yet the cylindrical epithelial character always remains. An infiltrative growth with destruction of the limiting membrane or deep proliferation of solid epithelial cones, as described by Selye *et al.* [1935] after administration of high doses of follicular hormone was not observed in our experiments. When the effect of the hormone ceases then the cornified non-nucleated cells in the vaginal smear disappear, the uterus atrophies and instead of the cornified epithelial cells nucleated epithelial cells appear: they are finally replaced by normal cylindrical epithelial cells.

Only in isolated cases and with esters of oestradiol having a very prolonged effect, were we able to observe in castrated rats a change of the single-layered cylindrical epithelium of the mucosa into a multiple-layered pavement epithelium as described by Selye *et al.* [1935], Pierson [1935], Grumbrecht [1935], Korenchevsky [1937], Szarka & Rechnitz [1938], Dessau [1938] etc.

We must mention that in our rats, even when using high doses of esters of oestradiol having very prolonged action, malignant tumours were never observed.

Note. After completing this work we became aware of a publication by A. Fischer [1938] entitled: "Ueber die biologische Wirkung der Follikelhormon-Derivate." Probably following on our own publications [Miescher, 1937; Miescher *et al.* 1937, 1, 2, 3; 1938, 1, 2], this author also investigated esters of oestrone and oestradiol, particularly oestradiol-17-mono-esters and determined, as we did, besides the threshold value in the oestrus test also the duration of the oestrus and the effect on the uterus. The oestrus threshold values ascertained on rats agree with those communicated by us. To determine the duration of oestrus, Fischer gave a single dose of 1γ to castrated mice and to ascertain the effect on the uterus he gave within 4 days 4 injections of 1.7γ each to young rats and weighed the dried uterus on the 5th day. Fischer did not obtain satisfactory results with the uterus test and therefore he recommends the seminal vesicle test.

As is shown from the comparison with our results the experiments made by Fischer are to be considered as inadequate for the judgment of the effect of sex hormone compounds. The efficacy of a substance cannot be judged on the results of tests with small doses alone. A proper conclusion regarding the total activity of such a compound can only be made when higher doses are also taken into consideration. In the uterus growth test (and moreover also in the seminal vesicle test) the time course of the swelling and the subsidence of the swelling of the organ after injection must be considered. When this test is carefully carried out there is, contrary to the opinion of Fischer, a certain parallelism between the duration of the effect in the oestrus test and in the uterus growth test.

SUMMARY

1. Nine aliphatic 3-mono-esters, ten aliphatic 17-mono-esters and the 17-monobenzoate of α-oestradiol were tested by subcutaneous injection into the rat by the oestrus test and uterus growth test and the results compared with those obtained with oestradiol-3-benzoate.

2. *Oestrus test*. In both series of esters containing a fatty acid radical the threshold values are, up to the octanoates, comparatively low and are similar to that of free α-oestradiol (0·4 γ). From the octanoates they increase rapidly but do not exceed 7 γ for the esters investigated. The threshold values of the 17-mono-esters are generally somewhat higher than those of the corresponding 3-mono-esters. A prolonged effect is obtained particularly with the higher esters. In this respect all the 17-esters investigated are much superior to the corresponding 3-esters; the 17-octanoate exhibiting the most intense and prolonged effect even with small doses.

3. *Uterus growth test*. In this test also 17-mono-esters, particularly the lower esters, prove to be superior to the corresponding 3-mono-esters. The results of experiments with oestradiol dipropionate and the 17-mono-octanoate on uncastrated young and on castrated adult rats agreed in an astonishing way.

4. *Histological observations*. The single-layered cylindrical epithelium changes under the prolonged influence of hormone into several strata of partly keratinized cells of very high cylindrical epithelium ("vertical keratinization"). An infiltrative growth with destruction of the limiting membrane or deep proliferation of solid epithelial cones did not occur with our method of testing. Malignant tumours were not observed. When the effect of the hormone ceased a return to normal followed.

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CLXX. THE ACID-SOLUBLE PIGMENT OF RED HUMAN HAIR

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THE pigment responsible for the distinctive colour of human red hair appears to be unknown. Text-books contain conflicting statements in regard to the question. Morse [1927] states that "red hair is a diluted black". If this is true, it becomes difficult to explain the many shades of brown which various samples of hair exhibit. On the other hand, Bodansky [1934] states: "In addition to melanin, the presence of lipochrome has been described in skin and hair, to which is attributed the characteristic red coloration which is often seen in hair." The only lipochrome discovered by Tutschku [1923] in horse hair, however, had a yellow-green colour and was present in hair of all shades except white. Neither Morse nor Bodansky quotes literature references in support of the statement contained in his text.

Zwicky & Almasy [1935] have reported that the alkali-soluble pigments of red hair, black hair and melanomas of horses cannot be distinguished spectroscopically. The present author does not agree with this conclusion, since inspection of their data shows that the ratios of the extinction coefficients of the black hair extracts and the red hair extracts are not constant. That is, the two spectral curves cannot be made to coincide at any concentrations of the pigments.

Neumann [1937] has recently made a study of the pigments of rabbit hair. Three pigments, described as yellow, brown and black, were found. The yellow pigment has the lowest molecular weight and the lowest nitrogen content while the black pigment has the highest molecular weight and nitrogen content.

EXPERIMENTAL

Extraction of acid-soluble pigment. Samples of red human hair were thoroughly washed with successive portions of $0.1 N$ NaOH, $0.1 N$ HCl and distilled water. The hair was then partly dried between filter papers, after which it was extracted with boiling $0.1 N$ HCl for periods varying from 3 to 10 days. During the first 2 days the refluxing solution remained colourless; from the third day on, however, coloration was present. The extract was concentrated by boiling and filtered to remove the hair residue and some protein which precipitated. The filtrates were clear; dilute solutions were coloured reddish yellow and more concentrated solutions were red-brown.

In order to demonstrate that the pigment extracted by this procedure was not produced by the chemical treatment, samples of black hair and of synthetic dopa-melanin (prepared by the oxidation of dopa [Arnow, 1938]) were refluxed separately with $0.1 N$ HCl for varying lengths of time. The red pigment was never produced in these experiments. Apparently black hair does not contain an acid-soluble pigment, and such a pigment cannot be produced from dopa-melanin by prolonged boiling with dilute HCl.

Preparation of synthetic pigment. Solutions of dopa-melanin in N NaOH were placed in 75 ml. test tubes. Air was bubbled through these solutions for several days. Excess HCl was then added and the precipitated dopa-melanin was removed by centrifuging and filtration. The resulting solution resembled in colour the extract of red hair.

The pigment produced in this manner from dopa-melanin appears to be an oxidation product. Solutions of dopa-melanin in N NaOH which are kept in containers evacuated with a water pump are stable more or less indefinitely. Similar solutions exposed to air slowly produce the acid-soluble pigment.

The experiments of Dulière & Raper [1930] indicate that melanin can be oxidized with oxygen in alkaline solution. On theoretical grounds, the production of melanin from tyrosine by tyrosinase should require 5 O per mol. of tyrosine. At pH 6 this value was found, by the above authors, but 5·23 O were used at pH 8. Moreover, "if a few drops of 30 % KOH were added to the solution in the experimental flask after all enzyme action had ceased, a further oxygen uptake was observed. This occurred outside the limits of pH at which tyrosinase is active and was probably due to production of oxidation products of melanin itself by atmospheric oxygen."

Comparison of natural and synthetic pigments. (1) Both pigments are soluble in acid, neutral and alkaline solutions. The term "acid-soluble pigment" has been used in this paper to distinguish the coloured compounds from melanin (and perhaps other hair pigments), which is insoluble in dilute mineral acid solutions.

(2) Both pigments exhibit more colour in alkaline solution than in equal concentration in acid solution. The degree of acidity or alkalinity can vary within wide ranges without affecting the colour, provided that the concentration of pigment is unaltered.

(3) The visible absorption spectra of the two pigments in either acid or alkaline solution agree qualitatively within experimental error (see Fig. 1). The absolute concentrations are not known, since neither substance has yet been isolated in pure form. It is possible, however, to adjust the solutions to equal concentrations as determined either with the spectrophotometer or with a Duboscq colorimeter. A Bausch and Lomb spectrophotometer was used in making the measurements recorded in Fig. 1. In accordance with Lambert's law, the extinction coefficient is defined by the expression,

$$\text{extinction coefficient} = \frac{1}{d} \log_{10} \frac{I_0}{I},$$

where I is the intensity of light after passage through d cm. of solution, and I_0 is the intensity of the light entering the solution. According to Beer's law, for any given wave-length this extinction coefficient is proportional to concentration, if d is maintained constant. The absorption vessels used in making these measurements were 2·5 cm. long.

Fig. 1 shows the increase in colour in alkaline solution, since the concentration of pigment is the same in both solutions. The colour in acid solution is not qualitatively identical with that in alkaline solution. This is illustrated in Fig. 1 by a plot of the ratio of the extinction coefficients in alkaline solution to those in acid solution as a function of the wave-length. This would result in a straight line parallel with the abscissa if the colours were qualitatively identical.

(4) When acid solutions of the two pigments are shaken with *n*-butyl alcohol, a portion of the pigment enters the alcohol layer. If the experiment is

repeated, substituting alkaline solutions for acid solutions, no pigment enters the alcohol layer.

(5) Solutions of both pigments pass through dialysing membranes (Visking casing).
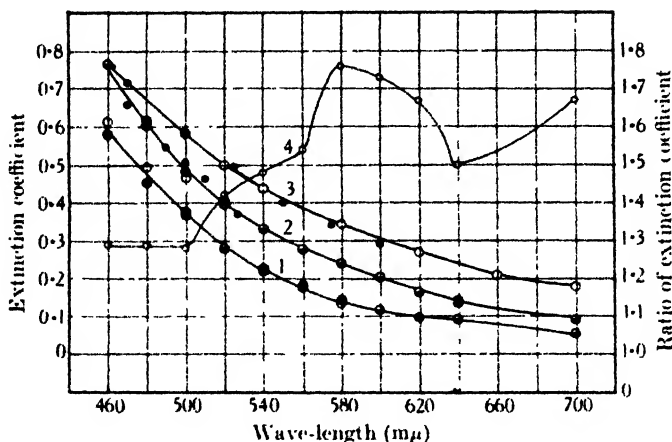
Fig. 1. Absorption spectra data.

Curve 1, pigments in hydrochloric acid solution.

    o solution of pigment extracted from red human hair.

    ● solution of synthetic pigment.

    • points calculated from data of Zwicky & Almasy [1935].

Curve 2, pigments in sodium hydroxide solution.

    o ⎫
    ● ⎬ As for curve 1.
    • ⎭

Curve 3, dopa-melanin and black horse hair pigment in alkaline solution.

    o dopa-melanin in 0·02N NaOH (1·37 mg. per l.).

    • points calculated from data of Zwicky & Almasy [1935].

Curve 4, ratio of extinction coefficients of the pigment of red human hair (and of synthetic pigment) in alkaline solution and in acid solution.

DISCUSSION

The evidence presented above suggests that the characteristic pigment of red human hair is an oxidation product of melanin. The strongest evidence of the identity of the synthetic and natural pigments is afforded by the spectral data. It is true that the evidence presented indicates only that the absorption spectra of the two pigments agree qualitatively with each other. It seems unlikely, however, that two chemically different substances could have the same qualitative spectra in both acid and alkaline solutions, particularly in view of the fact that the spectra are both quantitatively and qualitatively different in these solvents. In respect to the other physical properties recorded in this paper, the pigments also agree with each other.

The data of Zwicky & Almasy [1935], mentioned earlier, give some support to the present author's data. If the concentrations of the solutions studied by them are adjusted to those used in this paper by multiplying the extinction coefficients calculated from their data by a suitable constant, it is found that the spectral curve for the pigment of red horse hair agrees qualitatively with that for red human hair. This is indicated in Fig. 1, in which is also indicated the interesting fact that the spectral curve for the pigment of black horse hair

agrees qualitatively with that for dopa-melanin. An inspection of these graphs will show that it is impossible to make the curve for dopa-melanin (or black horse hair pigment) coincide with that for an alkaline solution of the pigment of red hair (or oxidized dopa-melanin) by multiplication of the values of the ordinates by a constant. For this reason, the author cannot agree with the conclusion expressed by Zwicky & Almasy, that "Das schwarze Pigment der einen Art, das Pigment roter Haare...erwiesen sich im gelösten Zustand als spektroskopisch ununterscheidbar". The expression "der einen Art" is used because a pigment whose absorption spectrum was obviously different from that of melanin was found in certain samples of horse hair.

Some protein is extracted along with the acid-soluble pigment, as indicated by the fact that the hair extract gives the usual protein colour tests, whereas solutions of the pigment prepared from dopa-melanin do not.

The fact that the colour of the pigments deepens in alkaline solution suggests that the compounds are weakly acidic in character. The increased colour would then be explained by the increased ionization in alkaline solution. This view is strengthened by the finding that butyl alcohol will extract them from acid aqueous solution, but not from alkaline aqueous solution.

SUMMARY

If red human hair is extracted with boiling 0·1 N hydrochloric acid, a red-brown pigment is obtained in solution. This pigment has the physical properties of a pigment prepared by the mild oxidation of dopa-melanin in alkaline solution. It is suggested that the distinctive colour of red hair is due to the presence in such hair of an oxidation product of melanin.

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CLXXI. THE EFFECT OF BODY STORES ON THE EFFICIENCY OF CALCIUM UTILIZATION

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MANY Ca and P balance experiments with animals and man have been carried out during the last 35 years. The resulting data are rather difficult to interpret because of much variation from unknown causes. It occurred to the writer that possibly the degree of Ca "saturation" of the tissues was a factor influencing the efficiency with which an individual utilized his dietary Ca.

It has been shown by Fairbanks & Mitchell [1936] that a low Ca content of the body brought about by subsistence on a diet poor in this element predisposes to a subsequent high retention of Ca when liberal amounts are furnished in the diet. In their experiment, the Ca content of the diet was 1·25 % in the experimental period which followed a preliminary period on a low Ca diet. The 1·25 % Ca level is so much in excess of the Ca requirement for maximum storage, however, that the animals were able to replenish their depleted Ca stores even with a low percentage retention. On the basis of the intake and balance figures given in their paper, the writer has calculated the average percentage retention for the animals that had been on the low diet in a previous period: this was found to be only 24 %. Hence, this experiment does not elucidate the question as to whether an individual becomes more efficient in the utilization of the available dietary Ca in times of stress.

In order to test this point it is essential that the Ca furnished in the diet in a test period, following a preparatory period on a low Ca diet, be below the requirement for maximum storage. It was felt that the point was of sufficient importance in connexion with the determination of the Ca requirements of man and animals to justify experimental enquiry.

FIRST EXPERIMENT

This experiment was conducted according to the paired feeding method. The food intake of a pair was restricted to the amount consumed by the member eating the less.

Six young hooded male rats were selected and paired, the two members of a pair being of the same litter and as nearly alike as possible. Four of the animals were 33 and two were 30 days old when they were started on the balance experiment.

A basal diet of the following composition was fed:

Yeast	5
Casein	5
Wheat gluten ...	15
Corn starch ...	60
Butter fat	12
Salt mixture ...	3

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The salt mixture was De Loureiro's [1931] with calcium phosphate omitted as adapted by Henry & Kon [1937], and was made up from B.D.H. "Analar" reagents.

This basal diet contained 0·10 % Ca and 0·19 % P; its P content was adjusted to 0·4 % by addition of K_2HPO_4. In the preliminary period the Ca was fed at two levels, namely 0·15 and 0·8 %, one member of a pair being fed the 0·15 % Ca diet, the other the 0·8 % Ca diet. The Ca content was regulated by addition of $CaCO_3$. In all cases the odd number rats received the low Ca diet in the preliminary period.

The diets were fed in four 7-day balance periods. The animals were kept in Hopkins metabolism cages made of stainless steel. At the end of each 7-day collection period the cages, beakers and funnels were rinsed in 5 % HNO_3 and the washings added to the urine. The urine was evaporated to dryness and ashed before being analysed.

The 4 weeks' preliminary period was followed by a 5 weeks' test period during which the Ca content of the diet was adjusted to the 0·4 % level for both members of a pair. At the end of the test period the animals were killed, the intestinal tract was removed and the carcass analysed for Ca and P.

Results

The food intake and gain in live weight are given in Table I. The average daily gain in live weight in the preliminary period was 1·5 g. for the animals on the low Ca diet and 1·7 g. for those on the high Ca diet; in the test period the live weight gain was almost the same in both groups, averaging 2·2 g. daily. The relatively low gain in the preliminary period was due to lack of appetite in the animals on the low Ca diet. There was not a single instance of refusal of food in the animals on the high Ca diet. On the low Ca diet, on the other hand, refusals occurred frequently, although the attempt was made not to feed more than the amount which would be eaten within 24 hr.

The food consumption improved in the test period and naturally a better growth was obtained. The figures for growth indicate a better food utilization on the high Ca diet, which was to be expected in the light of the studies by Toepfer & Sherman [1936].

Table I. *Food consumption and live weight gain (g.) in first exp.*

	Pair I		Pair II		Pair III	
	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
Initial live wt.	39	36	48	44	40	41
Final live wt.	153	150	167	169	165	175
Carcass wt.	133	128	145	147	143	153
Av. daily gain in preliminary period	1·3	1·5	1·4	1·6	1·8	2·0
Av. daily gain in test period	2·2	2·1	2·3	2·3	2·2	2·2
Daily food consumption in preliminary period	5·1	5·1	4·8	4·8	6·0	6·0
Daily food consumption in test period	8·7	8·7	9·2	9·2	9·3	9·3

The average Ca and P balances for the four weekly preliminary periods are given in Table II. On an average the animals on the low Ca intake stored 191 mg. Ca and 179 mg. P during the 4 weeks as compared with an average storage of 570 mg. Ca and 293 mg. P by their pair mates on the high Ca diet.

Table II. *Mean storage of Ca and P in first exp.*

	Rat no.	Ca					P				
		In-take mg.	Faeces mg.	Urine mg.	Stored mg.	Stored %	In-take mg.	Faeces mg.	Urine mg.	Stored mg.	Stored %
Weekly av. in pre-liminary period	1	53·2	2·5	1·7	49·0	92·1	142·0	12·9	86·9	42·2	29·7
	2	284·0	128·7	20·6	134·7	47·4	142·0	59·1	13·2	69·7	49·1
	3	49·9	12·6	1·4	35·9	71·9	133·0	14·2	85·6	33·2	25·0
	4	266·0	119·0	22·0	125·0	47·0	133·0	53·2	17·8	62·0	46·6
	5	63·0	3·2	1·4	58·4	92·7	168·0	13·2	96·0	58·8	35·0
	6	336·0	148·0	20·3	167·7	49·9	168·0	61·2	19·1	87·7	52·2
Weekly av. in test period	1	244·8	50·4	7·8	186·6	76·2	244·8	38·1	87·5	119·2	48·7
	2	244·8	116·6	8·4	119·8	48·9	244·8	71·5	88·3	85·0	34·7
	3	256·8	63·7	8·6	184·5	71·8	256·8	46·5	89·7	120·6	47·0
	4	256·8	137·2	21·3	98·3	38·3	256·8	74·6	83·9	98·3	38·3
	5	259·2	68·5	7·5	183·2	70·7	259·2	45·7	100·0	113·5	43·8
	6	259·2	109·2	8·1	141·9	54·7	259·2	65·5	94·4	99·3	38·3

At the start of the experiment two male rats similar to the experimental animals were analysed for Ca and P and found to contain 0·52 % Ca and 0·40 % P. In Table III will be found calculations of the Ca and P contents of the experimental animals at the end of the preliminary period, based on the assumption that these animals had the same initial Ca and P percentages as those of the two animals analysed. The average Ca and P contents of the rats on the low Ca diet worked out at 411 and 348 mg. respectively. The corresponding figures for the animals on the high Ca diet were 779 mg. Ca and 454 mg. P. The rats on the high Ca diet thus contained 90 % more Ca and 30 % more P than their pair mates on the low Ca diet, but in spite of the low Ca content of these latter animals, they seemed to be in excellent condition. The only difference between the pair mates which could be detected by the eye was a difference in the incisor teeth. The incisors of the depleted animals were opaque with white chalky patches. The incisors of the replenished animals were transparent with a uniform brownish lustre.

The balance data for the test period are given in Table II. The table shows that the depleted animals made up for previous shortage by a more efficient utilization of the Ca and P in the food. The depleted animals stored on an average

Table III. *Ca and P stored and Ca and P content of the carcasses at the end of first exp.*

Rat no. ...	1		2		3		4		5		6	
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca.	P
Initial content, mg.	203	156	187	144	250	192	229	176	208	160	213	164
Stored preliminary period, 28 days, mg.	196	169	539	279	143	133	500	248	234	235	671	351
Stored test period, 35 days, mg.	933	596	599	425	923	603	492	491	916	567	710	496
Total stored, mg.	1332	921	1325	848	1316	928	1221	915	1358	962	1594	1011
Found in carcass, mg.	1334	899	1292	842	1365	906	1370	906	1370	906	1538	1000
Difference, mg.	−2	+22	+33	+6	−49	+22	−149	−9	−12	+56	+56	+11
Difference %	0·1	2·4	2·5	0·7	3·6	2·3	10·9	1·0	0·9	6·3	3·6	1·1
Ca and P as % of net wt.	1·00	0·68	1·01	0·66	0·94	0·58	0·93	0·62	0·96	0·63	1·01	0·65
Ca/P ratio of minerals stored in preliminary period	1·2		1·9		1·1		2·0		1·0		1·9	
Ca/P ratio of minerals stored in test period	1·6		1·4		1·5		1·0		1·6		1·4	
Ca/P ratio of minerals in carcass	1·5		1·6		1·5		1·5		1·5		1·5	

924 mg. Ca and 589 mg. P during the test period as compared with 600 mg. Ca and 471 mg. P stored by their pair mates. During the entire experiment the storage was nearly equal in the pair mates. The average figures for the depleted animals are 1115 mg. Ca and 768 mg. P as compared with 1170 mg. Ca and 763 mg. P in the replenished ones.

The Ca and P contents of the animals as calculated from the balance figures showed good agreement with the Ca and P found by analyses of the carcasses, as will be seen in Table III. The Ca data for number 4 furnished an exception to this rule and showed a deviation of 10·9%. The deviation for the other animals ranged from 0·1 to 6·3%. In the preliminary period the animals on the high Ca intake lost a large percentage of the Ca in the faeces. The average for the three animals was approximately 45%. The animals on the low Ca intake excreted only very small amounts of Ca in the faeces; two of the animals excreted about 5%, the third about 25% of the food Ca in the faeces. In the amount of Ca lost in the urine there was also a marked difference between the pair mates. In the preliminary period the loss was from 6·1 to 8·3% of the intake for the animals on the high Ca intake. On the low Ca intake the corresponding figures were from 2·3 to 3·2%. Two of the animals on the low intake stored approximately 92% of the food Ca, the third stored 72%. On the high Ca diet the storage was from 47 to 50%.

The P excretion also showed a striking difference between the pair mates. On the low Ca diet the P was excreted mainly in the urine, on the high Ca diet mainly in the faeces. In the urine the averages were from 57 to 64% on the low Ca diet and only from 9 to 13% on the high Ca diet. In the faeces the excretion of P ranged from 36 to 42% on the high Ca diet and only from 8 to 11% on the diet low in Ca. The difference in P storage was about 20% in favour of the animals on the high Ca intake.

In the following 5 weeks' test period when the pair mates were fed the same diet in equal amounts, the depleted animals utilized the dietary Ca more efficiently than did their replenished pair mates. This was mainly brought about by better absorption from the intestine. The difference in the urinary Ca was comparatively slight, but this difference was in favour of the depleted animals too. The replenished animals lost from 42 to 53% of the Ca in their faeces as compared with 21 to 26% in the case of the depleted ones.

The P excretion in the urine was nearly equal for the pair mates. In the faeces on the other hand there was a difference of about 10% in favour of the depleted animals.

SECOND EXPERIMENT

This experiment was conducted in much the same way as the first one, the only differences being shorter balance periods and a lower Ca content of the

Table IV. *Food consumption and live weight gain (g.) in second exp.*

	Pair I		Pair II		Pair III	
	No. 7	No. 8	No. 9	No. 10	No. 11	No. 12
Initial live wt.	50	33	52	53	51	43
Final live wt.	146	153	150	159	159	161
Carcass wt.	135	142	138	147	147	148
Av. daily gain in preliminary period	2·0	2·7	1·8	2·0	2·0	2·5
Av. daily gain in test period	2·5	3·0	2·9	3·0	3·1	3·1
Av. daily food consumption in preliminary period	7·0	7·0	6·9	6·9	7·6	7·6
Av. daily food consumption in test period	10·4	10·4	10·2	10·2	11·0	11·0

ration during the test period. The preliminary period and the test period each lasted for only 3 weeks and the Ca level in the test period was adjusted to 0·25%. A carmine marker was administered at the end of the preliminary period in order to effect a better separation between the faeces from the two periods.

The six male rats selected for this experiment were 33 days of age at the start. The food intake and gain in live weight as shown in Table IV were higher than in the previous experiment. The animals in this experiment gained only slightly less in 6 weeks than the animals in the first experiment did in 9 weeks.

Results

The balance data are given in Table V. The average storage for the preliminary period was 212 mg. Ca and 154 mg. P on the low Ca diet. On the high Ca diet the average storage was 545 mg. Ca and 280 mg. P. Assuming that these rats had the same initial percentages of Ca and P as the two rats analysed in the first experiment, the difference in Ca and P contents of the rats on the high and the low Ca diets at the end of the preliminary period was found to be less than in the first experiment. The calculated figures show that the animals on the high Ca diet contained 60% more Ca and 25% more P than did their pair mates on the low Ca diet. An examination of the incisor teeth did not reveal such a striking difference as that found in the first experiment, although the incisors of the depleted animals were definitely poorer than those of the replenished ones.

Especially in the first week of the test period the depleted animals proved to be more efficient in the utilization of dietary Ca. The total Ca excretion in the faeces and the urine was more than three times as high in the replenished animals as in their depleted pair mates. The difference in the Ca excretion between the pair mates in the preceding period, however, was so large that if only a small amount of faeces from the diet of the preliminary period had been collected at the beginning of the test period, the Ca contained herein would easily account for the higher Ca content of the faeces from the replenished rats in the first week of the test period. The use of a marker does not entirely dispose of this possibility since the marker does not effect a perfect separation. The P excretion, however, strongly indicated that no excreta from the previous period had been collected in the test period. In the week prior to the change in diet the P excreted in the faeces was 5–6 times larger in rats on the high than in those on the low Ca diet. In the urine the reverse condition held true. On the low Ca diet 3–4 times more

Table V. *Mean storage of Ca and P in second exp.*

| | | | Ca | | | | | P | | |
| | | | | Stored | | | | | Stored | |
Rat no.	Intake mg.	Faeces mg.	Urine mg.	mg.	%	Intake mg.	Faeces mg.	Urine mg.	mg.	%
Weekly av. in preliminary period										
7	74·0	3·7	1·3	69·0	93·2	197·3	16·9	131·1	49·3	25·0
8	394·6	178·7	42·9	173·0	43·8	197·3	80·4	19·9	97·0	49·2
9	72·5	3·1	1·6	67·8	93·5	193·3	16·2	127·9	49·2	25·5
10	386·6	190·4	27·2	169·0	43·7	193·3	78·8	35·3	79·2	41·0
11	79·5	2·7	1·4	75·4	94·8	212·0	17·8	138·7	55·5	26·2
12	424·0	185·0	36·0	203·0	47·9	212·0	81·2	27·6	103·2	48·7
Weekly av. in test period										
7	182·5	14·0	2·6	165·9	90·9	292·0	26·7	140·0	125·3	42·9
8	182·5	22·5	6·6	153·4	84·1	292·0	26·9	134·8	130·3	44·6
9	179·2	10·8	2·6	165·8	92·5	286·6	24·7	126·9	135·0	47·1
10	179·2	25·2	9·2	144·8	80·8	286·6	30·3	136·2	120·1	41·9
11	191·6	10·7	3·4	177·5	92·6	306·6	25·7	145·7	135·2	44·1
12	191·6	20·0	7·1	164·5	85·9	306·6	28·6	143·1	134·9	44·0

P was excreted in the urine than on the high Ca diet. During the first week of the test period the pair mates excreted nearly equal amounts of Ca and P in the faeces and urine.

In the following 2 weeks the depleted animals stored only slightly more Ca than did their pair mates. The difference was mainly brought about by a smaller Ca loss in the faeces though the urinary Ca excretion also favoured the depleted animals. In P metabolism there was no significant difference between the pair mates.

The agreement between the Ca and P contents of the rats calculated on the basis of balance data and the Ca and P contents found by analyses of the carcasses was very close (Table VI). The maximum deviation was only 2·5 % for Ca and 4·0 % for P.

Table VI. *Ca and P stored and Ca and P contents of the carcasses at the end of the second exp.*

Rat no. ...	7		8		9		10		11		12	
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
Initial content, mg.	260	200	172	132	270	208	276	212	265	204	224	172
Stored preliminary period, 21 days, mg.	207	148	519	291	203	148	507	238	226	167	609	310
Stored test period, 21 days, mg.	498	376	460	391	497	405	434	360	532	406	493	405
Total stored, mg.	965	724	1151	814	970	761	1217	810	1023	777	1326	887
Found in carcass, mg.	990	712	1166	807	963	732	1198	801	1011	759	1300	891
Difference, mg.	-25	+12	-15	+7	+7	+29	+19	+9	+12	+18	+26	-4
Difference %	2·5	1·7	1·3	0·9	0·7	4·0	1·6	1·1	1·2	2·4	2·0	0·4
Ca and P as % of net wt.	0·73	0·53	0·82	0·57	0·70	0·53	0·82	0·54	0·69	0·52	0·88	0·60
Ca/P ratio of minerals stored in preliminary period	1·4		1·8		1·4		2·1		1·4		2·0	
Ca/P ratio of minerals stored in test period	1·3		1·2		1·2		1·2		1·3		1·2	
Ca/P ratio of minerals in carcass	1·4		1·4		1·3		1·5		1·3		1·5	

During the preliminary period on the low Ca diet the animals stored on an average 212 mg. Ca and 154 mg. P. The corresponding figures for their pair mates were 545 mg. Ca and 280 mg. P. In the test period the average storage was 509 mg. Ca and 396 mg. P for the depleted animals, as compared with 462 mg. Ca and 385 mg. P for their replenished pair mates.

In the test period the depleted animals stored 92 % of the Ca and 44·7 % of the P. The corresponding figures for their pair mates were 83·6 and 43·5. The replenished animals excreted about 6 % more Ca in the faeces and about 2·5 % more Ca in the urine than did their pair mates. It must be kept in mind, however, that the difference between the pair mates decreased after the first week of the test period.

DISCUSSION

Data reported in the literature indicate that 0·4 % Ca in the diet is inadequate for maximum storage when growth is normal. The rate of growth obtained in these experiments, however, was below normal and the possibility that the replenished animals were able to keep their tissues "saturated" with Ca must be considered.

The analyses of the carcasses support the view that the animals in the first experiment did keep their tissues "saturated" with Ca on the 0·4 % Ca level. The balance data on the other hand show a much smaller Ca content of the live

weight increase in the test period than in the preliminary period on the 0·8 % Ca level. Calculated on the basis of the balance data and gain in live weight the live weight increase contained 1·19 % Ca and 0·61 % P in the preliminary period. In the test period on 0·4 % Ca the live weight increase contained 0·78 % Ca and 0·61 % P. The high Ca figure for the preliminary period is of course partly brought about by the low initial Ca content of the animals, but even when allowance is made for the increase in Ca percentage due to increase in age, there is still a difference left between the two periods which may be explained as resulting from the inadequacy of the Ca level of the diet in the test period.

In the second experiment the Ca level was so far below the requirement that the replenished animals were rapidly depleted of Ca, which undoubtedly contributed to improved utilization as the test period advanced. The carcasses of the depleted animals contained 0·71 % Ca as compared with 0·84 % in the replenished animals. The corresponding figures for the first experiment were 0·97 and 0·98.

From the findings of the two experiments it seems justifiable to draw the conclusion that the degree of Ca "saturation" has an effect on the efficiency of Ca utilization. The efficiency is greater with depleted than with replenished stores. This must be taken into consideration when interpreting mineral balances in animals and man. Equilibrium or positive balance is not necessarily proof of adequacy of intake. It might be in part a reflexion of the level of previous mineral nutrition. In other words, an intake which will barely maintain equilibrium with depleted stores might not suffice to maintain equilibrium when the stores are filled. In this connexion one might naturally ask if it is desirable to keep the Ca stores filled. Our present knowledge is not sufficient to answer this question, but it does seem likely that Ca stores may be considerably reduced and Ca utilization consequently improved without any harmful effect to the individual.

Under the conditions of this experiment, the P excretion in the faeces was determined to a considerable extent by the amount of Ca present. In the preliminary period of both experiments, on the high Ca diet about 40 % of the ingested P was lost in the faeces and 8–10 % in the urine. The corresponding figures for the low Ca diet are 8–10 % in the faeces and 60–70 % in the urine. McGowan [1933] showed with rabbits that a shift in the P excretion from the urine to the faeces occurred after addition of $CaCO_3$ to the diet but that the retention was better with $CaCO_3$ than with dicalcium hydrogen phosphate or steamed bone flour. He claimed that the neutralization of gastric HCl by $CaCO_3$ or other basic salts was the cause of the lowered Ca and P absorption from the intestine. In the test periods of the present experiment it was not the neutralization of gastric HCl which caused an increased loss of P in the faeces. The pair mates were fed the same diet but none the less the replenished animals lost more Ca and P in the faeces than did their depleted pair mates. This fact seems best explained on the assumption that the large amount of Ca present in the faeces of the replenished animals due to reduced absorption interfered with the absorption of P by reason of the formation of relatively insoluble calcium phosphates. The experiment does not, however, exclude the possibility that the neutralization of the HCl of the stomach did play a part in the high P excretion in the faeces of the animals on the high Ca diet in the preliminary period.

Summary

1. The degree of Ca "saturation" of the tissues affects the efficiency of Ca utilization when Ca is furnished at levels below the requirement for maximum retention.

2. Under the conditions of these experiments the P excretion in the faeces tends to run parallel with the Ca excretion.

3. Lack of Ca in the diet affects the appetite adversely.

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CLXXII. DETERMINATION OF CALCIUM IN RAT URINE

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THE assay of parathyroid hormone by the method of Dyer [1937] requires the mass determination of Ca in small amounts of rat's urine. The methods given in the literature apply chiefly to human urine, of which large amounts are readily available, and involve incineration, which greatly limits their usefulness for mass analysis.

The chief objection to precipitating Ca oxalate directly from urine appears to have been the danger of simultaneous adsorption or precipitation of uric acid [cf. McCrudden, 1911–12]. This is not very probable in the case of rat or mouse urine, in which uric acid is replaced largely by allantoin, and could probably be avoided in any case by varying the concentration and pH of urine and the duration and temperature of precipitation. In the case of rat or mouse urine a further disturbing factor enters, namely, the invariable presence of protein in these urines, and this has, as will be seen later, a small but definite interfering effect.

The object of the present research was to determine whether, and under what conditions, the microtitration method of Halverson & Bergeim [1917] is applicable to urine, deproteinized or not, the results being compared with those obtained for ashed urine.

EXPERIMENTAL

(1) *Materials*

Rat and mouse urines were collected in glass metabolism cages of the type described by Dyer [1937], with groups of 5 rats or 10 mice in each cage; the daily output was made up with diluted acetic acid to 100 ml. Human urine was taken from healthy adults.

(2) *Methods*

The Halverson-Bergeim technique, as modified by Van Slyke & Sendroy [1929], was applied in all cases to the final determination of Ca. The following minor modifications were found desirable. The precipitate often has a tendency to adhere to the sides of the tube and to float on the surface of the liquid. This can be obviated by adding oxalate to the solution at 80° and allowing it to cool to room temperature; large crystals of Ca oxalate are thus obtained which readily settle. Three washings with 1:50 ammonia are preferable to two, as recommended by Van Slyke.

The filtered acidified urines were deproteinized by mixing 10 ml. of 20% trichloroacetic acid with 25 ml. of diluted urine (containing 0·4–0·8 mg. Ca), and centrifuging after 30 min.

In view of the destructive action of phosphates on platinum vessels, dry incineration is not a desirable procedure. Wet ashing with H_2SO_4-$(NH_4)_2S_2O_8$, by the method of Shohl & Pedley [1922], or with H_2SO_4-$KHSO_4$ gave much lower results than those obtained by direct precipitation, both for urine and for standard $CaCl_2$ solution. Since the only difference in the final solutions was the presence of Na_2SO_4, this was added in amounts of 0–1 g. to 10 ml. of standard solution containing 0·1235 mg. Ca, and Ca was determined as above. The amount recovered fell rapidly as the amount of $SO_4^=$ added exceeded 0·1 g., being only 0·06 mg., or 50 % of the actual value, with 0·9 g. $SO_4^=$. It follows that microdetermination of Ca is not possible under the given conditions of incineration.

Ashing with HNO_3, by the method of Dyer, gave results which were substantially identical with the theoretical results. Thus the Ca content of a standard solution was 0·3169 mg. by direct precipitation, and 0·3189 mg. after incineration. Applied to urine the method also gave satisfactory results, and the values so obtained were taken as representing the true Ca content of the urines.

Apart from protein, uric acid has been supposed to interfere with direct precipitation of Ca. The precipitates obtained from amounts of urine (rat, mouse or human) containing 0·15–2 mg. Ca were tested by the Folin-Denis reaction for uric acid, but in no case was a positive reaction obtained, even after standing for 24 hr.

A necessary preliminary to the study of conditions of precipitation from urine was to determine the effect of varying the conditions under which Ca was determined in standard solutions, namely, duration, temperature and pH of the precipitation, time of centrifuging and number of washings. The results, given in Table I, show that the degree of accuracy is the same whether precipitation takes place at 3° or at 37°, and that precipitation is complete after 3 hr. Varying the pH from 3 to 6 does not significantly affect the results and the same is the case when the time of centrifuging is varied from 5 to 15 min.

Finally, it appears from the last column that the precipitates should be washed with three 3 ml. portions of 1:50 ammonia. It is evident that the

Table I. *Effect of varying duration, temperature and pH of precipitation, time of centrifuging and number of washings. All tubes contain 0·1235 mg. Ca. The figures in parentheses refer to the number of determinations, of which the mean is given*

Temperature at which		Mg. Ca found with various intervals elapsing before centrifuging						Time of centrifuging min. at 3000 r.p.m.	pH	No. of washings
Oxalate added	Systems kept before centrifuging	40 min.	1 hr.	2 hr.	3 hr.	4 hr.	24 hr.			
19-21°	19-21°	0·1188 (5)	0·1196 (5)	0·1154 (3)	0·1188 (5)	0·1202 (3)	0·1181 (3)	15	4·5	3
19-21°	3°	0·1147 (2)	0·1198 (2)	—	0·1238 (2)	—	—	15	4·5	3
19-21°	37°	0·1188 (2)	0·1188 (2)	—	0·1268 (2)	—	—	15	4·5	3
80°	20°	—	—	—	—	0·1188 (2)	—	15	3	3
80°	20°	—	—	—	—	0·1233 (2)	—	15	3-4	3
80°	20°	—	—	—	—	0·1263 (4)	—	15	4-4·5	3
80°	20°	—	—	—	—	0·1239 (6)	—	15	4·5-5	3
80°	20°	—	—	—	—	0·1223 (2)	—	15	5-6	3
80°	20°	—	—	—	—	0·1256 (3)	—	5	4·5	3
80°	20°	—	—	—	—	0·1288 (2)	—	10	4·5	3
80°	20°	—	—	—	—	0·1225 (3)	—	15	4·5	3
80°	20°	—	—	—	—	0·1369 (2)	—	15	4·5	1
80°	20°	—	—	—	—	0·1268 (3)	—	15	4·5	2
80°	20°	—	—	—	—	0·1241 (3)	—	15	4·5	3

method is applicable over a wide range of conditions, which might be so selected as to exclude the interfering action of urinary constituents.

An analogous study was made of the optimum conditions for the determination of Ca in deproteinized rat urine. On the basis of these results the following method was adopted. The metabolism cage is rinsed with 5% acetic acid and the urine + washings are diluted to 100 ml. 10 ml. of 20% trichloroacetic acid are mixed with 25 ml. of filtered diluted urine and the solution is centrifuged after 30 min. (15 min. at 3000 r.p.m.). 5 ml. of the clear solution are diluted to 10 ml. in a calibrated conical centrifuge tube, and 1 ml. of 20% Na acetate is added, followed by 6 drops of bromocresol green indicator. 1 : 1 ammonia is then added drop by drop, with constant stirring, to a yellowish-green colour, indicating a pH of 4·5–5. The tubes are then placed in a water bath at 80°, and 1 ml. of 4% ammonium oxalate is added. After 5 min. the tubes are removed from the water bath, and are maintained at room temperature for $\not< 4$ hr., after which they are centrifuged (5 min. at 2500–3000 r.p.m.). The supernatant fluid is removed by suction, leaving 0·2 ml. of solution, the precipitate is dissolved in 2 ml. of N H_2SO_4 (5 min. at 100) and the solution is titrated at 70 with 0·01 N $KMnO_4$.

Table II. *Effect of varying duration and temperature of precipitation in deproteinized rat urine*

Temp. before centrifuging	Mg Ca found in 5 ml. urine, with a precipitation period of		
	1 hr.	2 hr.	3 hr.
3	0·0714	0·0704	0·0734
21	0·0765	0·0805	0·0754
37	0·0775	0·0815	0·0805

The effect of varying the duration and temperature of precipitation is shown in Table II: the values given represent the means of two determinations. It appears that higher values are obtained at 37° than at 3 , but that with a duration of precipitation of 3 hr. the difference is not significant. Over a pH range of 3·5–6·5 (Table III) the highest values were obtained at pH 4·25–4·55. At pH 6·5 phosphate crystals (probably $MgNH_4PO_4$. $6H_2O$) were formed in some cases, and these interfered with the collection and washing of the Ca oxalate precipitate. As in the case of standard $CaCl_2$ solutions, 3 washings of the precipitate were found to be essential. Varying the duration of centrifuging from 5 to 15 min. did not affect the results. Ca was determined in 2–10 ml. of deproteinized urine: the results, calculated for 5 ml. of urine, were 0·1157, 0·1110, 0·1082, 0·1128, 0·1131 and 0·1107, for 2, 3, 4, 5, 8 and 10 ml. of urine respectively, corresponding with maximum deviations from the mean of ± 0·012 mg.: this difference does not exceed the maximum error of the titration.

Table III. *Completeness of precipitation of Ca from deproteinized rat urine at varying pH*

pH	3·55	3·75	4·25	4·40	4·55	5·90	6·50
Mg. Ca	0·0735	0·0754	0·0886	0·0835	0·0825	0·0815	0·0775

A comparison of the results obtained with and without deproteinization (Table IV) shows that whilst the mean results do not in any case differ by more than the permissible error of titration, yet the differences between the individual determinations are greater in presence of protein, and the mean results are consistently lower.

Table IV. *Results of determining Ca in untreated and deproteinized rat urines.*
All values refer to 5 ml. of urine

No. of urine	Untreated urine		Deproteinized urine		Difference
	Mg. Ca found in				
1	0·087 0·080 0·077 }	0·081	0·092 0·088 0·088 }	0·089	0·008
2	0·118 0·110 0·112 }	0·113	0·118 0·117 0·124 }	0·119	0·006
3	0·166 0·160 0·153 }	0·159	0·163 0·165 0·165 }	0·164	0·005
4	0·086 0·087 }	0·087	0·098 0·102 }	0·100	0·013

A comparison of the results obtained for deproteinized and ashed rat urines (24 samples) gave a mean difference of 0·009 mg. in 5 ml. of urine in favour of the ashed urines. The difference is not significant, and it was hence concluded that incineration is not essential.

The results for mouse urine were not so satisfactory (Table V), as the differences between deproteinized and ashed urines in some cases exceeded 0·03 mg. In the case of human urines even greater differences were found, the values for ashed urine being from 0·015 to 0·060 mg. higher than for untreated urine. It may perhaps be concluded from these results that part of the Ca of human urine is not precipitable by oxalate and that under our conditions uric acid is not an interfering factor.

Table V. *Determination of Ca in mouse and human urines. The values given*
are the means of 3 determinations

No. of urine	Source of urine	Untreated urine a	Deproteinized urine b	Difference a−b	Ashed urine c	Difference b−c
		Mg. Ca found in 5 ml. of				
1	Mouse	0·267	0·303	− 0·036	0·270	+ 0·033
2	,,	0·272	0·265	+ 0·007	0·262	+ 0·003
3	,,	0·158	0·196	− 0·038	0·189	+ 0·007
4	,,	0·235	0·247	0·012	0·266	0·019
5	,,	0·287	0·289	− 0·002	0·307	0·018
6	,,	0·262	0·280	− 0·018	0·293	− 0·013
7	,,	0·315	0·349	− 0·034	0·374	− 0·025
						a−c
1	Human	0·325	– –	–	0·356	− 0·031
2	,,	0·435	– –	–	0·495	− 0·060
3	,,	0·728	–	—	0·772	− 0·044
4	,,	0·497	–	—	0·539	− 0·042
5	,,	1·683	–		1·713	− 0·030
6	,,	0·304	–	– –	0·319	0·015

SUMMARY

1. Calcium can be determined with satisfactory accuracy in small amounts of deproteinized rat urine by a modification of the method of Halverson & Bergeim. Incineration of the urine is not essential, but deproteinization is desirable.

2. Uric acid is not precipitated together with Ca oxalate, nor is it adsorbed by the precipitate. Higher values are given by incinerated than by untreated human urine.

3. Precipitation of Ca oxalate is inhibited by $SO_4^=$ in concentrations of over 1%. Methods of ashing involving the addition of H_2SO_4 are not applicable to the micro-determination of Ca in urine.

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CLXXIII. INVESTIGATIONS INTO THE METHOD OF ESTIMATING VITAMIN E

II. FURTHER OBSERVATIONS ON VITAMIN E DEFICIENCY AND IMPLANTATION

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THE response to vitamin E treatment cannot be graded satisfactorily. Attempts to make use of the size of a litter, while ignoring the viability of its individual pups, would seem to have little justification; nor can the observer always be certain that he has in fact observed all the pups, dead or alive, that have actually been expelled from the maternal organism. The simplest procedure seems, therefore, to treat the response as of the all or nothing kind, and it becomes necessary to define what we mean by the birth of a litter.

We assess as a positive response the appearance of any number of animals, from one upwards and of whatever viability, whether they are found alive or not; it is clearly impossible to know in every instance whether all members of a litter were actually breathing at the time of birth.

We were at one time faced with a further difficulty. On the 22nd or 23rd day after mating it would sometimes be found impossible to tell whether a resorption had occurred and the response was accordingly to be judged negative, or whether a small number of non-viable young had indeed been born, only to be eaten by the mother before the cage had been inspected. In the absence of any sign of young, such a response, which should by definition have been judged positive, would have been recorded as negative. Accordingly, all cages containing does that have shown positive implantation are now fitted, on the 19th or 20th day after mating, with a movable grid, of mesh about $\frac{3}{4}$ in., placed $2\frac{1}{4}$ in. above the cage bottom. The doe remains on this grid, but the young that are born fall through on to the cage bottom. They seldom survive, but it is in any event our practice to destroy them; our sole concern is to have objective evidence that they have come into existence. Even so, a small number of animals may be consumed by the mothers before they have ever been deposited on the cage bottom (or grid): no account can be taken of these, whether the grid is used or not. The phrase "expelled from the maternal organism" must be read as meaning completely expelled and actually deposited by the mother on the floor of the cage.

We take as a measure of response what we call the fertility rate, or the percentage fertility, that is, the number of litters, as defined above, in relation to the number of positive implantations expressed as a percentage. The use of the fertility rate is exemplified later in this paper.

In an earlier publication [1937] we have advocated the use of virgin rats as test animals for vitamin E. Apart from the time saved by avoiding a preliminary gestation-resorption, a further economy is achieved in both time and material, as virgin animals show a higher implantation rate than those (R.P. animals) that have already undergone a gestation-resorption.

These results have been confirmed on a further large number of animals, drawn from our colony of highly inbred Wistar (London Strain) albino rats. The results are shown in Table I. In Table II we have combined the results from animals discussed in our earlier communication and those upon which Table I is based, but have re-divided them into 3 groups, according as the implanted animals produced no litters, up to one litter for every five implantations or more than this number. It will be seen from Table II that, at all 3 levels of vitamin E dosage, virgin animals showed much higher implantation rates than did the R.P. animals; the figures also confirm our former observation that the percentage of implantations is not affected by the level of vitamin E dosage after positive mating.

Table I. *Implantation rates*

Type of animal	No. of animals showing positive mating	No. of implantations	Implantation rate (%)
R.P.	59	32	54
Virgin	542	469	87

Table II. *Implantation rates*

	R.P. animals	Virgin animals
Dosage 0 (no litters)	61% (115)	90% (221)
Dosage (to 20% fertility rate)	74% (42)	90% (147)
Dosage (>20% fertility rate)	56% (188)	87% (317)
Totals	59·7% (345)	88·6% (685)

The figures in parentheses indicate the numbers of animals mated.

In our previous paper we suggested as the most probable cause for the reduced implantation rate of R.P. animals that the process of gestation-resorption itself produces some permanent change in the animal's reproductive system. In order to test this point further, the following experiment has been carried out.

Twenty-five pairs of young female rats were placed at weaning on our basal diet, REES 1. When the animals came into oestrus, one of each pair was mated with a buck of known fertility, and mating was repeated if necessary until all 25 animals had shown the "plug" or the presence of live spermatozoa in the vaginal contents. Of these 25 animals, 24 showed the placental sign on the following 15th or 16th day; the 25th animal was re-mated and then became implanted. None of these 25 animals produced any living young, and all showed the symptoms of typical gestation-resorptions. They were all re-mated on showing oestrus again after the normal gestation period, and were then also given for 5 days a daily dose of 4·5 mg. of an extract prepared from wheat germ oil. Of these 25 R.P. animals, 15 showed failed implantation, giving the low implantation rate of 40%. Of the 10 implanted animals 5 had live litters, a "fertility rate" of 50%.

When the R.P. animals were about to be mated for the second time, their sisters were examined and also mated at the first normal oestrus. These were therefore virgin animals that had been submitted to the deficient diet REES 1 for some 3–4 weeks longer than is usual with our test animals, while they were waiting for their litter-mate sisters to be re-mated; their period on the diet, however, was identical with that of these R.P. litter-sisters. Of the 25 virgin animals, 21 showed positive implantations; this implantation rate of 84% is not far below the average for the whole of our recorded virgin animals. All 25 animals received the same dose of wheat germ oil extract as their R.P. sisters,

Table III. *Litter-mate comparisons*

Type of animal	Mated	Implanted	Fertile	Implantation rate %	Fertility rate %
R.P.	25	10	5	40	50
Virgin	25	21	16	84	76

and 16 of the 21 animals with positive implantations produced live litters, a fertility rate of 76 %. The results are summarized in Table III.

It seems certain that in groups of 25 animals the difference between 40 % implantation for R.P. animals, and 84 % implantation for virgin animals, is highly significant; the differences in implantation rates cannot be due to different periods of vitamin E deficiency, since both groups of animals received the diet for the same time. It is probable, though not certain, that the difference in fertility rates of the 10 R.P. animals and the 21 virgin animals is also significant, indicating that the R.P. animals have not only a lower rate of implantation, compared with virgin animals, but also a higher vitamin E threshold. The relatively small number of R.P. animals involved in this comparison, however, makes it impossible for this point to be conclusively demonstrated.

We have some contributory evidence for this apparent difference in threshold. A sample of carefully preserved wheat germ oil concentrate (unsaponifiable matter largely freed from sterols by freezing) was in use for the preparation of a standard dose-response curve in experiments that are being made the subject of another communication. When tested on R.P. animals, this concentrate gave the following results:

Dose mg.	Implanted	Live litters	Fertility rate %
105	10	6	60
230	9	8	89

For the preparation of the dose-response curve only virgin animals were used. The comparable figures for fertility rates were:

Dose mg.	Implanted	Live litters	Fertility rate %
22·5	39	34	87·2
11·25	44	15	34·1

For both the higher and the lower doses of these two groups, it appears that R.P. animals may require 6 times as much vitamin E as virgin animals, if they are to show the same fertility rate. This threshold ratio is the highest we have encountered: it is more usually in the neighbourhood of 4 or 5 to 1.

In the classical monograph of Evans & Burr [1927] occur some observations on the relation between vitamin E and implantation. 1 am much indebted to Prof. J. C. Drummond for calling my attention to these remarks. Evans & Burr wrote: "We must call attention, firstly, to the fact that between five and eighteen per cent of all cases of 'positive' matings in normal animals, i.e. those held upon natural foods, result in failure to implant. Secondly, animals maintained on a basal ration and used to demonstrate the incidence of the specific sterility disease should not show an appreciably higher proportion of failed implantations than do animals maintained upon natural foods[1]. In our records of somewhat over fifteen hundred positive matings in animals on natural foods, only 82 to 95 per cent are followed by implantations. A much lower implantation per cent denotes either peculiar food inadequacy different in nature from that due to low E, or denotes the presence of some special toxic factor or general constitutional debility[2]." The footnotes [1] and [2] above also deserve quotation in full. "[1] The reader may naturally inquire as to whether

failure to implant may not possibly represent a more advanced stage of the particular sterility disease due to lack of vitamine E. It would appear that a conclusively negative answer can be given here inasmuch as, although we now have evidence of the gradual depletion of the body in its residual stores of E and the consequent necessity in old animals of somewhat higher curative doses of wheat germ oil, for instance, than is essential in young ones on the same diet, we do not have evidence of added difficulty in implantation in such cases. Furthermore, and more decisive—many cases of persistent failed implantation were given wheat germ oil in excess daily for over a month without effect on implantation." "[2] We would especially inform the reader in some groups of animals reared and held on the basal ration, the ovulation, acceptance of coition and especially the implantation have been so deficient as to demand the rejection of most individuals of the group. It is only when these phenomena are normal that animals can be used for experiments calculated to show the presence or absence of vitamine E in any dietary regime." The authors then go on to discuss the nature of food deficiencies and special toxic factors.

Whatever effect such factors may have on implantation rates, they have been as far as possible excluded in our experiments, for both groups of animals received treatments identical except in the one variable under examination. It is not clear whether the quoted remarks of Evans & Burr apply only to virgin animals or not: if they do, their observations are consistent with our own.

One possibility must be mentioned, namely, that it is the occurrence of gestation as such, and not the pathological process of a gestation-resorption, to which is to be attributed the reduced implantation rate. This, as we mentioned in our earlier publication [1937], is contrary to experience. For example, a very large colony of animals of the same strain as our own stock was maintained by the Macaulay Laboratory at the Institute of Animal Genetics in Edinburgh University for some years. Detailed records of mating are available for a considerable number of those animals [Wiesner, 1938]. They indicate that animals under 150 days old, mated for the first time, had a fertility rate of about 89 %. The animals were receiving a normal stock diet, and no question of vitamin E deficiency therefore arises: any failure in fertility of animals that had undergone positive mating must have been almost entirely due to failed implantation. The fertility of animals of similar age, positively mated after the birth of a litter, also showed about 89 % fertility, indicating no change in implantation rate.

In spite of this evidence, we have planned a further crucial experiment to distinguish between the effects on implantation rate of a "normal" gestation and the pathological condition of gestation-resorption. Meanwhile, we feel more than ever inclined to the view that toxic by-products from the resorbed embryos have inflicted some lasting damage on the uterine or other mechanism of implantation.

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CLXXIV. THE PHYSIOLOGICAL PROPERTIES OF ASCORBIC ACID

I. AN EFFECT UPON THE WEIGHTS OF GUINEA-PIGS

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THE technique of paired feeding has been extensively used in nutritional investigations, particularly in those dealing with amino-acids and with vitamin B_1. The advantage of this procedure has been clearly indicated by Mitchell & Beadles [1930]. Its chief merit is that the otherwise variable factor of food consumption may be controlled. It has been known for some years that guinea-pigs, maintained on diets deficient in ascorbic acid, lose appetite, consume less food and diminish in weight. As in the case of vitamin B_1 it seemed reasonable that some of the symptoms manifest in these deficient animals might be caused by inanition. An extensive search of the literature has shown that only on one reported occasion has paired feeding been undertaken in studies on ascorbic acid. Anderson & Smith [1924], using a small number of animals and employing foods as sources of ascorbic acid, found that guinea-pigs fed a basal diet with supplements, the total amount being isocaloric with the consumption of scorbutic animals, were able to maintain body wt. at a higher level. The present investigation deals with an effect of *l*-ascorbic acid upon body wt. in paired feeding.

METHODS

The basal diet, which was designed to be deficient only in ascorbic acid, was a modification of that used by Coward & Kassner [1936]. Its composition was as follows:

	%
Wheat bran	45
Crushed oats	25
Dried skim milk	25
Brewers' yeast	2
Cod liver oil	1
$CaCO_3$	1
NaCl	1

The dried milk was heated in open trays at 110° for 2 hr. with frequent stirring to destroy its content of ascorbic acid. This diet without supplement produced normal growth in young rats and, when supplemented with ascorbic acid, in young guinea-pigs.

Guinea-pigs reared in the Connaught Laboratories' colony, weighing 200–230 g., were used. They were housed in individual screen-bottom cages. Water was supplied *ad lib.* and food was given in detachable metal boxes deep enough to prevent spilling. The room temp. was regulated at 72–74° F.

For the first week of experimental feeding the basal diet was supplied *ad lib.* and each animal received daily oral doses of 5–10 mg. *l*-ascorbic acid in freshly prepared aqueous solution. Daily wt. records were kept. It was assumed that,

at the end of this preliminary week, all animals were saturated with ascorbic acid since daily doses of 2 mg. have been found to give maximal weight increase in this strain of guinea-pigs. This preliminary period also served to accustom the animals to changes in housing and diet. By the end of the week most animals showed a normal weight response: a few which failed to do so were discarded.

The animals were then divided into 3 groups, each individual of which corresponded as closely as possible to individuals in the other 2 groups. The division was made on the basis of wt., sex and rate of wt. increase during the preliminary week. The first group, designated as "normal", received the basal diet *ad lib.* and 2 mg. ascorbic acid daily. The second or "control" group was given the basal diet only. The third or "paired" group received 2 mg. ascorbic acid daily and an amount of food equivalent to that which had been consumed by the control group on the previous day. Animals in this group retained a good appetite throughout experimental feeding and ate all the food furnished. The animals were weighed daily after ascorbic acid was given to the normal and paired groups and before being fed. Daily records were kept of the amounts of food given to and consumed by each animal.

The duration of the experiment was 21 days after the preliminary week, the average survival time of the paired and control animals. If for any reason an animal in either the control or paired group died before this time, its wt. and those of the corresponding animals were not included in the averages which are reported.

EXPERIMENTAL RESULTS

Observations have been made on 8 series. In some of these there was no normal group. Concordant results have been secured throughout. Since results
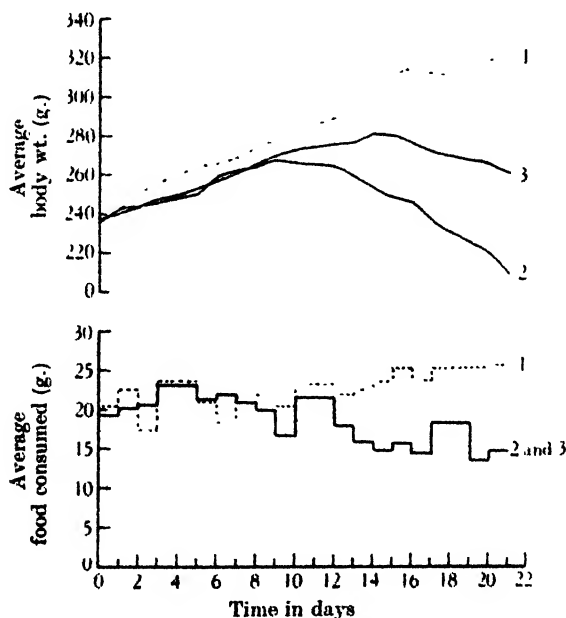
Fig. 1. Body weights and food consumption. 1, normal group; 2, control group; 3, paired group.

in good agreement have been given by all of 94 pairs in control and paired groups, data on one series only will be given. The accompanying figure shows

the curves of average wt. for three groups of 10 animals each and also shows the average food consumption for each group.

DISCUSSION

Following a period of 10–12 days, during which body stores of ascorbic acid are presumably exhausted, control animals lose appetite and diminish in wt. until death occurs. When food is furnished *ad lib.* a continuous supply of ascorbic acid causes an increase in wt. This response has been used for the quantitative estimation of ascorbic acid by Bracewell *et al.* [1930], Harris & Ray [1932], Hou [1936] and by Coward & Kassner [1936]. Ascorbic acid prevents a loss in wt. in animals paired with the control group. At the end of 3 weeks there has been in every case a significant difference in wt. between animals receiving isocaloric intakes.

A loss of appetite in guinea-pigs deprived of ascorbic acid has been commented upon by other observers. Anorexia is as characteristic of a deficiency of ascorbic acid as it is of vitamin B_1 deficiency. The supply of ascorbic acid to the paired and normal groups prevented the loss of appetite. Appetite can be maintained by as little as 0·1 mg. ascorbic acid per guinea-pig per day.

There are several likely explanations of the difference in wt. between two animals given isocaloric intakes, one of which received ascorbic acid while the other did not. The difference might be due to alterations in water balance, in absorption of food, or in metabolism. Observations on these three factors are being conducted and will be reported later.

SUMMARY

After 21 days of paired feeding young guinea-pigs receiving a scorbutic diet plus ascorbic acid are significantly heavier than animals of the same initial weight given only the basal diet. Since the difference in weight cannot be due to difference in food intake, it is believed to be due to alterations in metabolism, waterbalance and food absorption caused by a lack of ascorbic acid.

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CLXXV. ISOMERIZATION OF CAROTENOIDS

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It is of considerable interest to establish the limits within which the chromatographic method can be applied without danger of chemical alteration taking place in the column.

In the field of the carotenoids we were brought up against this problem [Zechmeister & Tuzson, 1938] in isolating the pigments of some fruits. The flesh of the water melon (*Cucumis citrullus*) in particular, which is rich in lycopene, sometimes gave extracts furnishing a single, very strong and homogeneous lycopene zone; in other experiments, however, carried out with the same raw material, the adsorbate showed a second layer below the lycopene ("*neolycopene*"), which could be washed down by means of benzene and light petroleum more easily than lycopene and differed markedly in its brownish yellow colour from the reddish lycopene zone.

A closer examination of the discrepancy between our various experiments showed that the character of the chromatogram depended on the time which elapsed between the extraction of the tissues and the beginning of the Tswett analysis. Fresh solutions furnished a single lycopene layer, older ones two coloured zones on the same part of the column; analogous results were obtained with solutions of pure crystalline lycopene. Light was thrown on these experiments by the fact that a freshly prepared solution of lycopene obviously changes its optical properties when kept. Further, this spontaneous alteration can actually be measured by means of colorimetric and spectroscopic determinations. The Tswett technique thus only furnishes a convenient demonstration of an effect already present in the solution.

The electronic configuration of the chromophore contained in the lycopene molecule becomes much less stable when the compound is dissolved. If the solution is kept at room temperature it can be easily shown that the colour intensity gradually decreases and that the extinction maxima slowly wander towards the shorter wave-lengths. Such changes must have been frequently observed by earlier workers but were probably ascribed to autoxidation. It cannot indeed be denied that simultaneous oxidative reactions do take place in the solution even under practically anaerobic conditions.

We find that autoxidation of lycopene leads partly to a colourless material and partly to coloured substances which are readily adsorbed on the highest section of the column and which cannot be reconverted into lycopene. On the other hand, *neolycopene* is always adsorbed below the lycopene zone and partly reverts to lycopene, if its zone is cut out and the elutriated substance is kept in a suitable solvent. The following experiments illustrate the processes indicated.

(*a*) A solution of 1·54 mg. lycopene (chromatographically homogeneous) in 20 ml. benzene was kept for 24 hr., diluted with light petroleum and chromatographed on $Ca(OH)_2$. Two layers were obtained, containing 1·26 mg. lycopene and 0·13 mg. *neolycopene*. A similar experiment gave after 3 days 1·02 and 0·31 mg. respectively.

(b) 1·57 mg. pure *neo*lycopene in 10 ml. benzene gave after 4 hr. 0·05 mg. lycopene and 1·49 mg. *neo*lycopene: after 24 hr. 0·08 and 1·15 mg. and after 2 days 0·12 and 1·13 mg.

If the solution is heated, equilibrium between lycopene and its isomeride is reached in a much shorter time. As can be seen from Table I, 5 min. boiling under reflux is sufficient to cause noticeable effects and within 30 min. about half the lycopene undergoes rearrangement in benzene. A very similar result is obtained by heating *neo*lycopene in benzene (Table III).[1]

In the course of extended experiments we came to the conclusion that many former authors must have actually worked with solutions which had come to equilibrium in the course of the usual laboratory operations such as concentration, heating etc. Since *neo*lycopene is transformed into ordinary lycopene by crystallization, it is understandable that its very existence has been overlooked. We may also mention that in some earlier experiments carried out by Dr Cholnoky in this laboratory, delay in the crystallization of and decrease in the colour intensity of certain lycopene solutions were observed, which could not at that time be explained.

Isomerization reactions among carotenes were first detected by Gillam & El Ridi [1935] and thoroughly described in two subsequent papers [Gillam & El Ridi, 1936; Gillam *et al.* 1937]. In the course of this extended and interesting study *pseudo*-α-carotene and *neo*-α-carotene were isolated in pure state. The formation of these new compounds was explained by the action of the adsorbent on carotenes. In such way repeated adsorptions must lead to increasing quantities of the isomeride. Without belittling the importance of the work of Gillam *et al.* we maintain that the isomerization of β-carotene is in all probability as spontaneous as that of lycopene. We claim that in no case is the phenomenon caused by the adsorption process and we are not able therefore to agree with the statement that "the analytical process itself affects the substances which it is designed only to separate".

In view of the earlier work of Gillam & El Ridi, our experiments with β-carotene were confined to the attempt to obtain sufficient information concerning the possible role of the adsorbent. As may be seen from the figures given in Table IV, the isomerization of β-carotene is directed by the same factors as the rearrangement of lycopene, though in the former case the reaction proceeds much more slowly. We find calcium hydroxide to be the most suitable adsorbent for such investigations, but since Gillam & El Ridi generally used alumina, a series of experiments was carried out with this adsorbent. Using the materials available to us we find that if a sample of β-carotene has once reached chromatographic homogeneity, little or no *pseudo*-α-carotene appears when the adsorption is subsequently repeated, provided that no considerable time elapses between each experiment and further, that all elutriations etc. are carried out not far from room temperature. Under these conditions we are unable to confirm the statement of Gillam & El Ridi that the amount of the newly formed isomeride increases with successive adsorptions.

Similar observations were made with various solutions of kryptoxanthin (Tables V–VI).

Finally we may briefly discuss the question of the natural occurrence of the new isomerides. Working under the established conditions, it was observed that rapidly-prepared and chromatographed extracts (*ex* tomatoes, water melon, berries of *Tamus communis* etc.) yielded perfectly homogeneous lycopene zones.

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[1] Preliminary experiments seem to show that both isomerides are much more rapidly interconverted at room temperature in the presence of a small quantity of iodine.

*neo*Lycopene was only formed if the same extracts were kept for some time. Already after 2 hr. at 20° a small amount of *neo*lycopene could be demonstrated in the column. The red fruits of *Actinophleus Macarthurii* (from Java) which were conserved in ammonium sulphate for 2 years, gave only a trace of *neo*lycopene. It is obvious that in these cases lycopene occurs in the tissue under conditions which inhibit isomerization and which from this point of view may be compared with the crystalline state.

Gillam & El Ridi [1936] claim that the isomerization of β-carotene is accelerated by the presence of some impurities: "Thus the carotene fractions of butter or blood serum...are separated readily into two zones of pigments by a single adsorption...whilst with pure crystalline β-carotene it requires much longer washing or more usually elution followed by another adsorption to bring about the isomerization." We suggest that these observations could well be explained either by the original presence of *pseudo*-α-carotene in the butter and blood serum pigment or by formation of it during the lengthy course of the preparation.

EXPERIMENTAL

(Partly in collaboration with Miss 1. BERGER)

Lycopene

Benzene solutions of crystalline lycopene (from various sources) were diluted with light petroleum (B.P. 60–80°), poured through a column of calcium hydroxide (25 × 5 cm.) and developed with a mixture of benzene and light petroleum 3 : 1. The main zone was cut out and eluted with benzene-alcohol. The latter was washed out and the remaining solution dried with Na_2SO_4 and evaporated rapidly *in vacuo* (bath temperature 40–45°). Under such conditions no isomerization takes place as was proved in special experiments. Working with small quantities the operation takes only 5 to 10 min.

The residue was dissolved in benzene, chloroform or acetone. If light petroleum were to be used in subsequent boiling experiments, the above evaporation could be omitted, the benzene being eliminated from the column by washing with sufficient quantities of light petroleum: the lycopene zone was then eluted with petroleum plus alcohol and the washed and dried eluate used directly for the boiling. Before proceeding to the latter an aliquot part of the solution was again chromatographed in order to check its absolute homogeneity. 20 ml. of each solution were boiled under reflux in an all-glass apparatus and the decrease of the colour intensity established by comparing directly the heated and the original solutions in a Leitz micro-colorimeter. The absorption maxima were measured with a Zeiss spectroscope.

Subsequently the cooled solutions were diluted with light petroleum and introduced into a calcium hydroxide column. (If acetone had been used, it was first eliminated by washing the solution.) In each case the chromatogram was developed with benzene and light petroleum and washed with a liberal quantity of the latter. A very good separation into two main zones was obtained, these being accompanied by much smaller layers on the upper part of the adsorbent.

The lycopene and *neo*lycopene zones obtained after 30 min. boiling were eluted with light petroleum-alcohol and, after elimination of the alcohol, compared with the Kuhn & Brockmann [1932] standard in the micro-colorimeter. As the tints of the two isomerides are different (lycopene reddish, *neo*lycopene brownish) it is advisable to use the solution of 145 mg. azobenzene in 100 ml. 96 % alcohol for lycopene and a 10-fold dilution thereof for *neo*lycopene. The results are summarized in Table I.

Table I. *Partial conversion of lycopene into* neolycopene

Solvent	mg. lycopene in 10 ml. of the original solution	Time of boiling min.	Spectrum mμ		Colorimetric value in % of the original	Amount of neolycopene formed in % of the total pigment in the column
Benzene	0·68	0	522,	487	100	
,,	0·68	5	519,	484	87	
,,	0·68	15	518,	485	87	
,,	0·68	30	519,	485	82	55
,,	0·69	0	522,	487	100	
,,	0·69	5	519,	485 ·	91	
,,	0·69	15	519,	484	84	
,,	0·69	30	519,	484	81	50
,,	1·25*	0	521,	485	100	
,,	1 25	30	519·5, 484		89	22
Chloroform	0·72	0	518,	484	100	
,,	0·72	5	517,	483	89·5	
,,	0·72	15	517,	482	87	
,,	0·72	30	516·5, 483·5		76	39
,,	0·82	0	518,	484	100	
,,	0·82	5	517·5, 484		86	
,,	0·82	15	517·5, 483·5		78·5	
,,	0·82	30	517·5, 482·5		73	36
Acetone	0·40	0	505·5, 475		100	
,,	0·40	5	505,	474	95·5	
,,	0·40	15	504·5, 473		90·5	
,,	0·40	30	504 5, 473		89·5	18
,,	0·54	0	505·5, 475		100	
,,	0·54	5	505,	474	96·5	
,,	0·54	15	504·5, 473		90	
,,	0·54	30	504,	473	83·5	20
Light petroleum	0·40	0	504·5, 472·5		100	
,,	0·40	30	502·5, 470		86·5	44
,,	0·28	0	504,	472	100	
,,	0·28	30	502,	470·5	85 5	42

* This solution was not boiled but kept at 56˚.

neoLycopene

This compound is much more easily soluble than lycopene itself and could not be obtained in crystalline form. Attempts to crystallize it from carbon disulphide-alcohol or benzene-methanol always led to samples which showed no perceptible difference from ordinary lycopene. The absorption bands of neolycopene show a displacement towards shorter wave-lengths (Table II).

Table II. *Absorption maxima of lycopene and* neolycopene *(mμ)*

Solvent	Lycopene			neoLycopene		
Carbon disulphide	547,	505,	475	536,	498, 466	
Benzene	522,	486,	445	512,	479, 450	
Chloroform	516,	479·5, 434·5		512,	478, 447·5	
Acetone	506,	475,	446	499·5, 468, 439		
Light petroleum	506,	474,	445	499·5, 468, 439		
Alcohol	504·5, 472·5, 445·5			500,	469, 439	
Ether	503·5, 471·5, 443·5			496,	466, 438	
Methanol	500,	470,	441	497,	467, 436·5	

On heating, *neolycopene* solutions, the colorimetric value of the liquid increases with considerable velocity (Table III) and the polyene which at first shows no evidence of heterogeneity separates into two well-defined zones on

Table III. *Partial conversion of neolycopene into lycopene*

Solvent	mg. *neolycopene* in 10 ml. of the original solution	Time of boiling min.	Spectrum mμ	Colorimetric value in % of the original	Amount of lycopene formed in % of the total pigment in the column
Benzene	~1	0	513·5, 580	100	
,,	~1	15	518·5, 487	135·5	
,,	~1	30	519·5, 484·5	153	~42

calcium hydroxide. The chromatogram is typical for the equilibrium mixture which may be obtained by starting from ordinary lycopene under the same conditions; the lycopene zone yielded a sample which gave no depression of M.P. when mixed with pure lycopene (*ex* tomatoes).

For the purposes of this work it was necessary to have a rough estimate of the colorimetric value of *neo*lycopene, i.e. the amount had to be established which, when present in 1 ml. light petroleum, shows the same intensity as 14·5 mg. azobenzene in 100 ml. alcohol. Since *neo*lycopene has not hitherto been obtained crystalline, the chromatographically homogeneous and colorimetrically matched solution was evaporated to dryness. The *neo*lycopene content was about 0·003 mg. 1 ml., showing that the compound is much less coloured than ordinary lycopene, for which the corresponding figure is 0·002 mg. ml.

β-Carotene

The isomerization of this polyene takes place more slowly than that of lycopene. A solution of 50 mg. pure substance in 200 ml. light petroleum was kept for 24 hr. at room temperature and showed, on a calcium hydroxide or alumina column which was washed with the above solvent, only a slight zone of *pseudo*-α-carotene, the amount of which was about 1–2 % of the total carotene. The boiling experiments which are summarized in Table IV were carried out as

Table IV. *Partial conversion of β-carotene into pseudo-α-carotene*

Solvent	mg. β-carotene in 10 ml. of the original solution	Time of boiling min.	Spectrum mμ	Colorimetric value in % of the original	Amount of pseudo-α-carotene formed in % of the total pigment in the column
Benzene	1·04	0	496, 464	100	
,,	1·04	5	496, 464	100	
,,	1·04	15	494·5, 462·5	90	
,,	1·04	30	495, 463	88	18
,,	1·43	0	496·5, 465	100	
,,	1·43	5	496, 464	97	
,,	1·43	15	495, 463	92	
,,	1·43	30	495·5, 462·5	92	21
Chloroform	0·86	0	495, 462	100	
,,	0·86	5	495, 462	98	
,,	0·86	30	494·5, 461·5	95·5	
,,	0·86	60	494, 461·5	93	11
,,	0·90	0	495, 462	100	
,,	0·90	5	494·5, 461·5	98	
,,	0·90	30	494·5, 462	97	
,,	0·90	60	494·5, 461·5	97	12
Acetone	1·33	0	486, 455	100	
,,	1·33	30	485·5, 453	~100	~0

described above, with the sole difference that the chromatogram was developed by means of pure light petroleum.

In a fairly long series of experiments we tried to establish whether the quantity of the isomeride increases or not from one adsorption to the next; the following data are typical.

(a) A solution of 100 mg. pure β-carotene in 150 ml. light petroleum (B.P. 60–80°) was chromatographed on alumina (mixture of 3 parts of Al_2O_3 Merck and 1 part standardized alumina, 25×5.7 cm.) and the column washed first with 1 l. light petroleum and then with 4 l. light petroleum plus 5 % benzene. Traces of by-products fixed on the upper part of the column, as also a very small amount of *pseudo-α-*carotene, were neglected. We eluted the β-carotene zone with light petroleum-alcohol. Then the liquid was washed, dried and poured through a new column. The *pseudo-α-*carotene layer of this second chromatogram was much smaller than before, and it disappeared totally when a third chromatographic analysis was rapidly carried out.

(b) An analogous experiment with calcium hydroxide gave us a small *pseudo-α-*carotene zone. In this case, however, even the second chromatogram was composed exclusively of a single carotene layer and in spite of abundant washing no isomeride appeared.

Kryptoxanthin

As may be seen from Table V, this polyene-alcohol exhibits a tendency to spontaneous isomerization which, under similar conditions, is greater than that of β-carotene. Solutions of *neo*kryptoxanthin may easily be converted into the

Table V. *Partial conversion of kryptoxanthin into neokryptoxanthin*

Solvent	mg. krypto-xanthin in 10 ml. of the original solution	Time of boiling min.	Spectrum mμ	Colorimetric value in % of the original	Amount of neokrypto-xanthin formed in % of the total pigment in the column
Benzene	0·86	0	496, 463	100	
,,	0·86	5	495, 462	96·5	
,,	0·86	15	494·5, 461	92	
,,	0·86	30	493·5, 461	90	28
,,	2·04	0	496, 463	100	
,,	2·04	5	495, 462·5	97	
,,	2·04	15	494, 462	91	
,,	2·04	30	493·5, 461·5	88·5	30
Chloroform	0·97	0	494, 462	100	
,,	0·97	5	493·5, 461	95·5	
,,	0·97	15	493, 461	95·5	
,,	0·97	30	493, 461	94	21·5
Acetone	1·14	0	487, 455	100	
,,	1·14	30	485, 454	92	15

Table VI. *Partial conversion of neokryptoxanthin into kryptoxanthin*

Solvent	mg. neokrypto-xanthin in 10 ml. of the original solution	Time of boiling min.	Spectrum mμ	Colorimetric value in % of the original	Amount of kryptoxanthin formed in % of the total pigment in the column
Benzene	~1	0	491, 460	100	
,,	~1	5	492, 461	103	
,,	~1	15	493, 461	106·5	
	~1	30	493, 461	110	46·5

equilibrium mixture with kryptoxanthin (Table VI). The absorption maxima of pure *neo*kryptoxanthin solutions are given in Table VII.

Table VII. *Absorption maxima of kryptoxanthin and* neo*kryptoxanthin* ($m\mu$)

Solvent	Kryptoxanthin	*neo*Kryptoxanthin
Carbon disulphide	518, 482	510, 478
Benzene	497, 464	490·5, 459
Chloroform	494, 462·5	490·5, 458
Acetone	486, 454	480·5, 449
Light petroleum	484·5, 451·5	478, 447
Alcohol	484, 452	479, 448

SUMMARY

1. Solutions of chromatographically pure lycopene $C_{40}H_{56}$, β-carotene $C_{40}H_{56}$ or kryptoxanthin $C_{40}H_{56}O$ undergo, when kept at room temperature, a partial isomerization which manifests itself in the decrease of the colorimetric value and in the displacement of the absorption maxima towards shorter wavelengths.

2. The speed of this spontaneous isomerization, which tends towards an equilibrium, increases on heating.

3. The interconversion is reversible.

4. Partly isomerized solutions always give two distinct layers in the Tswett column: the phenomenon is not caused by the adsorption experiment itself, but is already present in the solution. Experiments now in progress might show how far *cis-trans* changes are responsible for it [cf. Gillam *et al.* 1937].

Finally we wish to thank Dr A. E. Gillam for having kindly checked some of our experiments with lycopene. This work was aided by grants from the Duke of Eszterházy and from the Széchenvi Society.

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CLXXVI. COLORIMETRIC DETERMINATION OF SUBSTANCES CONTAINING THE GROUPING —CH$_2$.CO— IN URINE EXTRACTS AS AN INDICATION OF ANDROGEN CONTENT

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IN the expectation that a measure of endocrine activity in the human patient might be given by the level of excretion in the urine of compounds related to the male hormones, attention has recently been directed to the extraction of urinary constituents possessing androgenic activity and their measurement. As an alternative to biological assay, chemical methods have been applied. Zimmermann [1935] first suggested that steroid sex hormones could be determined quantitatively by the use of *m*-dinitrobenzene, which gives a red colour in presence of alkali with compounds containing an active methylene group. A year later [Zimmermann, 1936] he published a modified method which was applied to pure compounds (androsterone, testosterone, oestrone and equilin), and to urine extracts. Wu & Chou [1937] described a modification of the method, and used it on urine extracts with androsterone as a reference substance. Oesting & Webster [1938; cf. Oesting, 1937] used the Zimmermann technique, and roughly correlated their figures with the comb-growth produced in capons by inunction of the same extracts on the comb.

None of these investigators has considered simultaneously both capon assay and colorimetric assay in terms of pure hormones, and there was thus an obvious gap to be filled before it would be possible to test the colorimetric assay as an indicator of the androgenic activity of urine extract in terms of international units. The correlation of the figures obtained in this way would be expected to give data which, in addition to testing the value of the colorimetric assay on the assumption that excretion of androgenic activity was diagnostically significant (as has been done by Oesting & Webster), would also throw light on the chemical nature of the androgenic substances excreted in the urine, providing clues to the metabolism of steroid hormones, and giving a tangible chemical property which would serve as a guide in the analytical investigation of urine extracts.

As part of a scheme of work on the determination of hormones in blood and urine, undertaken under the auspices of the Hormones Committee of the Medical Research Council, investigations of methods of extraction of androgens from urine and their assay on capons have been in progress at this Institute for some time. These have been extended to a comparison of colorimetric and capon assays, and, on the chemical side in particular, to attempts to improve the sensitivity of the colorimetric method and to investigate its specificity.

The account of experimental work which follows deals in turn with the description of the modified colorimetric method finally adopted, a study of some of the many factors influencing the reaction, an investigation of the behaviour

of various methyleneketones of steroid and other types, and the correlation of colorimetric assay with capon assay for urine extracts from normal and certain abnormal clinical types.

EXPERIMENTAL

Reagents. (1) *Routine method of colorimetry of androsterone*

(a) *Alcohol.* Ordinary commercial "absolute" alcohol is used, the only further specification being that it should not have a content of aldehyde exceeding 0·0025 %.

(b) m-*Dinitrobenzene.* A well-crystallized and fairly pure material (we used B.D.H. "extra pure", M.P. 89–89·5´) is taken and further purified as follows: 20 g. are dissolved in 750 ml. of 95 % alcohol warmed to 40´ and 100 ml. of 2N NaOH are added. After 5 min. the solution is cooled, and 2500 ml. of water are added. The precipitated m-dinitrobenzene is collected on a Büchner funnel, washed very thoroughly with water, sucked dry and recrystallized twice in succession from 120 ml. and 80 ml. of absolute alcohol. The material must be well crystallized in almost colourless needles, M.P. 90·5–91´. Admixture of a 1 % alcoholic solution with an equal vol. of aqueous 2N NaOH should give no colour after an hour. The reagent is a 2 % w/v solution of this material in absolute alcohol. It is stored in a brown, stoppered bottle in the dark, and is stable for 10–14 days. In the actual colorimetric measurement (see below) the control solution, without methyleneketone, should give a pale straw colour having a value of $E_g = 0·20$–$0·21$ in a 1 cm. cell compared with alcohol.

(c) *Potassium hydroxide.* The reagent solution is 2·5N KOH in absolute alcohol. 9 g. of KOH (B.D.H. "Analar" pellets) are dissolved with mechanical stirring in 50 ml. of absolute alcohol, and the solution filtered through a hardened paper (Whatman No. 50) at the pump. The concentration is checked by titration of 0·5 ml. with 0·1N H_2SO_4 (methyl orange indicator) and the solution diluted with alcohol if necessary, to bring it within the limits of 2·48 and 2·52N. The solution is stable for 2–5 days if stored in a refrigerator. It must be discarded as soon as the faintest colour is perceptible.

Mode of operation

Test tubes used for the reaction must have been cleaned with nitric and chromic acid mixture. Into one tube, to serve as "blank", are measured out in succession, from 1 ml. pipettes graduated to 0·01 ml., 0·2 ml. of alcohol, 0·2 ml. of m-dinitrobenzene solution and 0·2 ml. of KOH solution. The solution of the test substance is measured out into a second tube, and then there is added sufficient alcohol to make the volume up to 0·2 ml., followed by the reagents. The time of adding the KOH is noted. The tubes are well shaken to disperse the dense KOH solution, lightly stoppered, and placed in a water bath kept at $25 \pm 0·1´$ by means of a thermo-regulator. The tubes are shielded from all but dull, diffused light by a screen. After an hour, 10 ml. of alcohol are added to each tube, and the contents mixed and transferred to the cells of the colorimeter, which are then closed by microscope coverslips.

Measurement of the colour

Use of the Spekker photoelectric absorptiometer. In this instrument the light absorbed by a coloured solution is measured photoelectrically by a null method. The light transmitted by the test solution falls on a photoelectric cell, the current from which is balanced by current from a second cell with an adjustable iris in front of it. The test solution is then replaced by the blank or control

solution, and balance again obtained by closing in the path of the beam a shutter actuated by a wheel with a logarithmic scale, from which an "absorption coefficient" (E) is read off directly. Using light which has passed through a selective filter, a value of E for a limited range of wave-lengths is obtained. In this work, cells of 1 cm. thickness have been employed, with an Ilford "spectrum green" filter (max. transmission at 5200 Å.). As a routine, readings (denoted by E_g) on one pair of solutions are made in triplicate: in our experiments these differ by not more than 0·01 over the range $E_g = 0·1–0·5$, which is the most suitable for measurement. It is desirable to complete the measurement within 5 min. of the dilution with alcohol. The methyleneketone content of the test substance in terms of androsterone is then determined from the absorptiometer reading (E_g) by reference to a calibration curve constructed from measurements with androsterone. All the recorded measurements, unless otherwise specifically stated, have been made by comparison of the test solution with a control blank containing reagents only.

Calibration curve. The androsterone used as standard was a purified sample with m.p. 184–185° (corr.). In the beginning of this work measured amounts of an alcoholic solution were evaporated to dryness in a stream of nitrogen on a

Table I. *Calibration of Spekker absorptiometer*

Androsterone (mg.)	0·025	0·05	0·075	0·10	0·15	0·20	0·25	0·30
E_g (a)	—	0·205	—	0·395	0·545	0·66	0·74	0·80
E_g (b)	0·10	0·19	0·275	0·365	0·50	0·60	-	
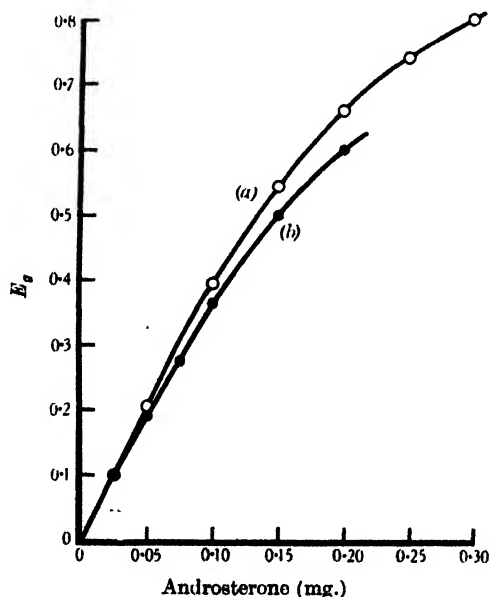
Fig. 1. Calibration curves for the reaction: androsterone + m-$C_6H_4(NO_2)_2$ + KOH (test solution – blank) on Hilger Spekker absorptiometer with Ilford "spectrum green" filters.

water bath, and alcohol and reagents added to the residue. It was soon found that loss occurred owing to the volatility of androsterone, and after this the androsterone was measured out in the form of appropriate amounts (0·1–0·2 ml.) of 0·025–0·1 % solutions in absolute alcohol, made up to a total volume of 0·2 ml.

Using the precautions specified above, it has been possible to reproduce colorimetric values for certain weights of androsterone within comparatively narrow limits, but there is a residue of variation which we have not been able to control. In all probability this is due to impurities in the alcohol and the *m*-dinitrobenzene. In view of the number of factors influencing the reaction, as described in detail below, the agreement is satisfactory. Over a period of seven months, during which the calibration curve has been frequently checked, the maximum variation has been between the limits shown in Table I and curves (*a*) and (*b*) in Fig. 1.

At the upper limit suggested for readings, $E_g = 0.5$, the difference, in terms of weight of androsterone, is 12 %. This difference does not, of course, represent the error in the determination of unknown materials when the position of the calibration curve with a new set of reagents is confirmed in the routine manner by colorimetry of a known amount of androsterone. Thus, ten measurements with 0.1 mg. of androsterone over a period of 12 days with two apparently similar batches of reagents gave a value of 2.0 % for the standard error of the absorptiometer reading, corresponding to a standard error of 2.7 % in the estimation of the weight of androsterone after reference to the calibration curve.

Use of a visual colorimeter of plunger type. With the co-operation of Mr S. W. Stroud, working in the Courtauld Institute of Biochemistry, Middlesex Hospital, an investigation has been made of the use of a Leitz two-stage colorimeter in the colorimetry of androsterone and urine extracts. With this instrument observations can be made with light which has passed through selective filters. It is only necessary to choose a colour standard. Potassium permanganate solution has an absorption spectrum which is very similar in the green, except for its banded character, to the net absorption spectrum of androsterone and reagents less that of reagents alone. Permanganate solution plus blank can therefore be matched approximately against test solution plus water, and accurate matching is possible in the light transmitted by the Leitz "yellow-green, 531" or "green-yellow, 551" filters; the former, transmitting light nearer the centre of the absorption band, gives higher readings.

The colorimeter is set up with test solution, prepared in the usual way, in the top cup and water in the bottom cup on one side, and, on the other side, blank in the top cup and $N/1000$ KMnO$_4$ in the bottom cup, the last being in a 20 mm. layer. The top plungers, which move together, are adjusted until the beams through the two sides are matched, and the depth of the layer is then read off on the scale.

A calibration curve was constructed with weights of androsterone from 0.05 to 0.225 mg. and the data are given in Table II and plotted in Fig. 2 in the form of weight of androsterone against the reciprocal of the scale reading.

Estimations made in this way on six urine extracts were compared with estimations on the Spekker absorptiometer, and gave the following results in terms of colorimetric equivalent, in mg., of androsterone per day's output, the pairs of figures being from Leitz and Spekker instruments, respectively: (*a*) 7.5, 8.0; (*b*) 7.0, 7.5; (*c*) 15.5, 15.3; (*d*) 15.7, 15.8; (*e*) 1.5, 1.75; (*f*) 24.0, 22.5. The agreement is fairly good. The disadvantage of this technique with the Leitz instrument is the impossibility of making absolute determinations of the absorption in the violet. As explained below, this may be an important matter in the recognition of occasional urine extracts in which the chromogen is abnormal, and the value of E_g alone bears no relation to the maximum of the absorption band.

Table II. *Calibration of Leitz colorimeter*

Androsterone (mg.)	0·05	0·075	0·10	0·125	0·15	0·175	0·20	0·225
Scale reading (531 filter)	38·3	23·4	17·1	14·3	11·9	10·0	8·7	8·4
Scale reading (551 filter)	31·5	19·3	14·6	11·3	9·2	7·6	7·3	6·6
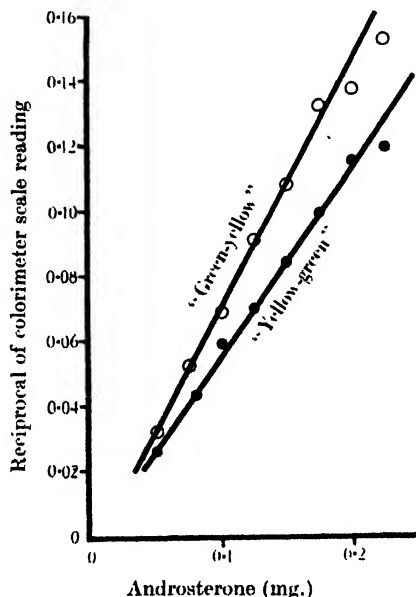
Fig. 2. Calibration curves for the reaction: androsterone + $m\text{-}C_6H_4(NO_2)_2$ + KOH (test solution + water/blank + 0·001N KMnO$_4$) on Leitz colorimeter with "green-yellow" and "yellow-green" filters.

Application to urine extracts

Colorimetric measurements have been made on the neutral fractions of urine extracts made by the method described by one of us [Callow, 1936, 1] with minor modifications. The acid and phenolic fractions contain ketonic material, and must always be removed. The neutral fraction is usually taken up in alcohol at a concentration such that 1 ml. contains the equivalent of either 0·1 l. of original urine, or 0·1 day's output, and 0·1 ml. of this solution is taken and diluted with 0·1 ml. of alcohol for colorimetry as described above. The colour of the urine extract itself is not sufficient to interfere with the determination. An unusually highly coloured extract gave a value of $E_g = 0·01$ when diluted in the proportion of 0·01 l. urine to 10·6 ml. Moreover, treatment with charcoal generally produces no appreciable change in the colorimetric reading (Oesting [1937] found that androgenic activity was not lost in this way). As in the case of androsterone, evaporation of alcoholic solutions of urine extracts, unless very carefully done, entails loss not only by volatilization but also by creeping, and this method of taking a known amount was abandoned.

The absorptiometer reading is converted, by reference to the calibration curve, into an equivalent in weight of androsterone, which is then expressed as mg. of "sterone" per litre or per day's output of urine. We have coined the term "sterone", to avoid giving the wrong impression that this value represents the biological and not merely the chromogenic equivalent of androsterone.

(2) *Factors influencing the reaction with androsterone*

General. In preliminary trials it was immediately found that, with un-purified m-dinitrobenzene, the technique of Wu & Chou [1937] gave a higher colorimetric reading than that of Zimmermann [1936] with the same amount of androsterone; the former method was therefore chosen as the starting-point for investigation of the factors influencing the sensitivity. Certain of these had already been studied by Zimmermann (Z.) and by Wu & Chou (W.C.), namely: (1) the solvent (Z., W.C.), (2) concentration of alkali (Z., W.C.), (3) concentration of m-dinitrobenzene (Z., W.C.), (4) time of measurement after mixing (Z.), (5) temperature of development (Z., W.C.), (6) illumination (Z.). With so many variables concerned (and this list is not exhaustive) it was to be expected that further systematic work would lead to an increase in the sensitivity of the reaction. Moreover, by choosing conditions under which the effect of variation in any factor was changing most slowly, the reproducibility of the readings might be increased.

Solvent: purity and amount. Small amounts of aldehyde in the alcohol cause increased absorption in both the test and the blank solutions, but more in the latter, so that, as a result of over-compensation, the value of test minus blank decreases with increase in the aldehyde. The figures in Table III were obtained using our routine technique with 0·1 mg. of androsterone. The absorptiometer with the "spectrum yellow-green" filter was used for the determination of aldehyde in samples of alcohol by means of the colour produced with Schiff's reagent. In separate experiments the same alcohol, of known aldehyde content, was used for dissolving the androsterone, the m-dinitrobenzene and the potassium hydroxide.

Table III. *Influence of acetaldehyde on E_g with 0·1 mg. androsterone*

% MeCHO	Test minus blank	Blank minus EtOH	Test minus EtOH (calc.)
0·0005	0·395	0·11	0·505
0·0017	0·37	0·17	0·54
0·0025	0·35	0·195	0·545
0·005	0·31	0·27	0·58

The effect of altering the total amount of solvent in the reaction mixture was tested as follows: portions of 0·25 mg. of androsterone were dissolved in 0·15, 0·22 and 0·26 ml. of alcohol, and to each were added 0·2 ml. of 2 % m-dinitrobenzene solution and 0·19 ml. of 2N alc. KOH. Controls were made up to the same total volumes, viz. 0·54, 0·61 and 0·65 ml. The values of E_g after dilution at the end of 1 hr. were respectively: 0·65, 0·62 and 0·57. The effect of using 95 % alcohol instead of absolute alcohol as a diluent after incubation was to reduce the value of E_g for 0·1 mg. of androsterone from 0·365 to 0·35. With the earlier technique the difference was greater. The specifications in the routine method of amount of alcohol, and of content of aldehyde and water, were adopted as giving a sensitive reaction whilst setting limits attainable in practice.

Purity and concentration of m-*dinitrobenzene.* Ordinary m-dinitrobenzene gives a red colour with potassium hydroxide. This has been attributed to the presence of dinitrothiophene [Meyer & Stadler, 1884] which is detectable in a quantity of 0·1 μg. [Stadler, 1885] by this reaction. Nitration of "thiophene-free" benzene gave a product which still gave a colour with alkali, and we therefore followed the method of Willgerodt [1892] for removal of chromogenic material, details of which are given above. This method must be adhered to

strictly, for overheating, or prolongation of the alkali treatment, gives poorly crystallized products of low M.P. which are useless for colorimetry. One such sample, after repeated crystallization from alcohol, then ethyl acetate, yielded yellow crystals, sparingly soluble in alcohol, M.P. 148° (soft at 145°), identified as 3:3'-dinitroazoxybenzene (lit. M.P. 143°, 146·5°). (Found (micro-analysis by Dr G. Weiler): C, 50·36; H, 3·08; N, 19·9. Calc. for $C_{12}H_8N_4O_5$: C, 50·0; H, 2·8; N, 19·45 %.)

The effect of impurity in the m-dinitrobenzene on the value of E_g is shown by the figures in Table IV, dealing with the variation of E_g with amount of KOH when different samples of m-dinitrobenzene were used, the "purified" samples having passed through the alkali process.

The effect of the amount of m-dinitrobenzene used was investigated at two stages of the development of the final method. We first confirmed Wu & Chou's

Table IV. *Variation of* E_g *with amount of KOH*

Wt. of androsterone (mg.)	0·25	0·25	0·10	0·10	0·05
m-Dinitrobenzene ...	Unpurified	Purified	Unpurified	Purified	Purified
Vol. of 2N alc. KOH (ml.)			E_g		
0·09	0·32	—	—		
0·12	0·47	0·365			0·12
0·15	0·54	—	—	0·28	
0·16	—	0·53	—		—
0·19	0·56	0·63	—		0·165
0·20	—	—	0·32	0·34	
0·25	0·51	0·66	0·34	0·365	0·20
0·275	—	—	0·345		
0·30	—	0·61	—	0·34	0·20
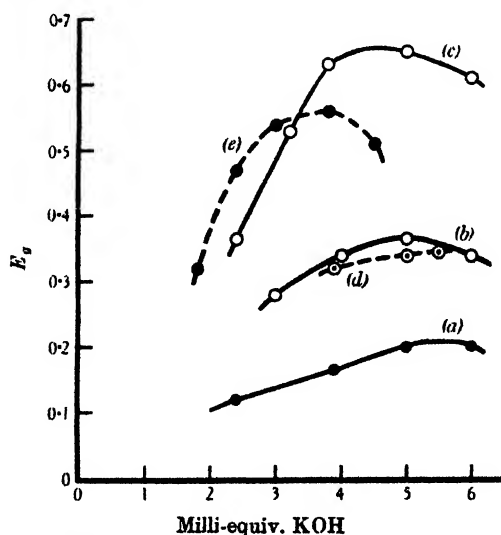
Fig. 3. Variation of E_g with amount of KOH. Curves (a), (b), (c): purified m-$C_6H_4(NO_2)_2$; 0·05, 0·1 and 0·25 mg. androsterone. Curves (d), (e): unpurified m-$C_6H_4(NO_2)_2$; 0·1 and 0·25 mg. androsterone.

observations that low values were obtained when small amounts of m-dinitrobenzene were used. In the final form of the reaction, 0·1 mg. of androsterone in 0·1 ml. of alcohol with 0·2 ml. of 2·5N KOH and varying amounts of 2 %

m-dinitrobenzene, viz. 0·14, 0·18, 0·20, 0·22 and 0·24 ml., made up to a total volume of 0·6 ml. with alcohol, gave the following values of E_g: 0·365, 0·36, 0·365, 0·375, 0·36. There was thus no systematic variation outside the limits of experimental error over this range of concentrations.

Effect of amount of potassium hydroxide. Series of tests were carried out in which androsterone was dissolved in $(0·41 - x)$ ml. of alcohol, 0·2 ml. of 2% alcoholic m-dinitrobenzene added, and then x ml. of $2N$ alcoholic KOH (x being varied from 0·09 to 0·3 ml.). Table IV gives the values of E_g for three series, one with an unpurified sample of m-dinitrobenzene, and these are shown graphically in Fig. 3.

The figure demonstrates particularly clearly the effect of purification of the m-dinitrobenzene. The proportion of KOH at which the absorption is maximal varies not only with the quality of the m-dinitrobenzene, but also with the amount of androsterone. The choice of 5 milli-equiv. of KOH in the standard method seems a satisfactory compromise, but the amount is evidently critical.

Effect of temperature and time of development. In general agreement with Wu & Chou, it was found that the absorption of the mixture diluted after 1 hr. increased by about 3% with a rise of 1° in the temperature of development.

With the technique modelled on that of Wu & Chou, it was found that with variation of the time of development the value of E_g rose to a maximum at about 1 hr., as shown by the series of figures in Table V, and there is no appreciable difference between 60 and 65 min.

Table V. *Variation of E_g with time of development of reaction mixture*

Time of development (min.)	0·05 mg. androsterone	0·10 mg. androsterone (a)	0·10 mg. androsterone (b)	0·10 mg. androsterone (c)	0·25 mg. androsterone (early technique)
30	0·17	0·315	- -	--	0·45
45	0·20	0·35	--	--	0·51
50		-	0·355	0·35	-
60	0·20	0·39	0·375	0·37	0·61
65	--	--	0·375	---	-
70		---	0·37	0·36	——
75	0·19	0·39	-	- -	0·60
90		0·365	- -	-	---
105	0·18	0·34	-	---	-
120	0·16	0·35	--	——	——

Effect of light. When the reaction mixture was developed in bright sunlight the value of E_g for 0·25 mg. of androsterone was 0·31 as compared with $E_g = 0·61$ with development in dull, diffused daylight. On the other hand, further diminution of the illumination had no effect: the value $E_g = 0·60$ was obtained after development in the dark. (Early technique.)

Stability of colour after dilution. The colour fades slowly after dilution of the reaction mixture, and the fading appears to be accelerated by exposure of the solution to light or air. In diffuse daylight and with closed absorptiometer cells the values of E_g for 0·1 mg. of androsterone were 0·37 immediately after dilution, unchanged after 25 min., and 0·325 after 4 hr.

Similarly with 0·25 mg. of androsterone, when the solutions were poured back into test tubes and replaced in the thermostat in between measurements, the values of E_g immediately after dilution, and after 30 and 60 min., were 0·61, 0·57 and 0·52 respectively.

Remaining sources of error. A potential source of variation is water in the alcohol or in the potassium hydroxide. This factor has not been investigated.

The hidden sources of error responsible for the residue of uncontrolled variation probably include this, the quality of the m-dinitrobenzene and manipulative errors. The presence of small amounts of cholesterol has no effect. The presence of digitonin reduces the colour: 0·1 mg. of dehydroandrosterone and 0·5 mg. of digitonin gave a value of E_g corresponding to 0·08 mg. No errors due to alteration of the filters or of the photoelectric cells have been detected in control measurements of the absorption of an "orange" glass filter made over a period of a year.

(3) Specificity—the reaction with other compounds

Spectral colorimetry. Zimmermann [1936], using a Pulfrich photometer, plotted curves which were a rough indication of the absorption spectra of the reaction mixtures of androsterone, oestrone, testosterone and creatinine, using

Fig. 4.

Fig. 5.
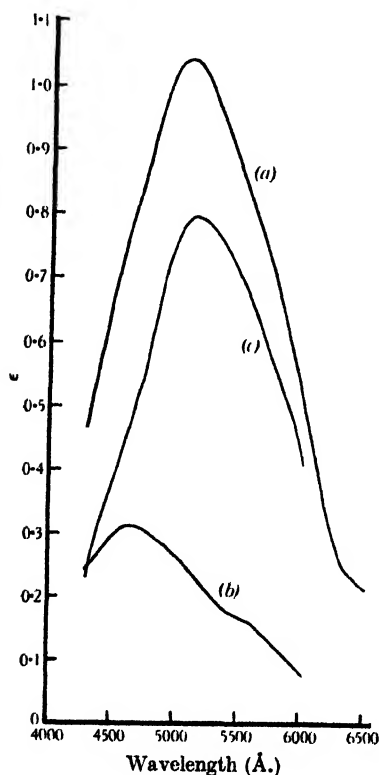
Fig. 4. Absorption spectra of (a) reaction mixture, 0·1 mg. of androsterone $+ m$-$C_6H_4(NO_2)_2 +$ KOH, and (b) of reagents alone, each against EtOH, with (c) calculated curve for test solution - blank. Hilger-Nutting spectrophotometer; 2 cm. cells; photometry of photographs at intervals of (a) $\epsilon = 0·05$, and (b) $\epsilon = 0·02$.
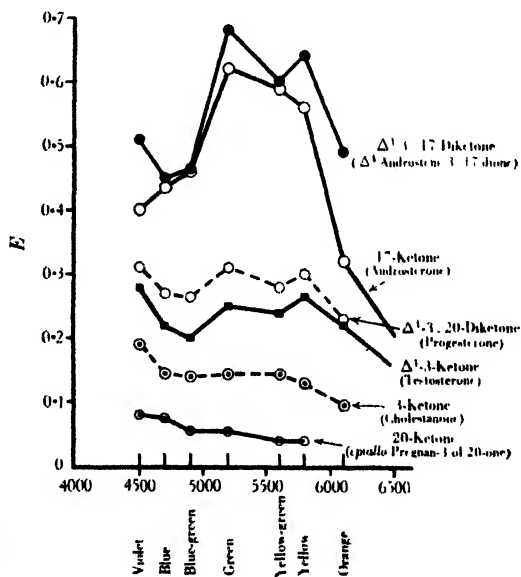
Fig. 5. "Absorptiometric spectra" with absorptiometer and Ilford "spectrum" filters of the reactions of typical steroid ketones $+ m$-$C_6H_4(NO_2)_2 +$ KOH.

measurements of the absorption within a limited range of wavelengths by inserting Zeiss "S" filters. We have used a similar method on a number of substances with the Spekker absorptiometer and Ilford "spectrum" filters. The

maxima of transmission of these filters, as given by the makers, occur at the following wave-lengths: violet, 4300 Å.; blue, 4700 Å.; blue-green, 4900 Å.; green, 5200 Å.; yellow-green, 5500 Å.; orange, 6000 Å. The range of transmission is of the order of 500–600 Å., except with the violet and orange filters, which are less selective. Measurements through these filters are designated below by the symbols E_v, E_b etc.

In the case of the reaction with androsterone, an accurate photographic measurement of the absorption spectrum has been made with a Hilger-Nutting spectrophotometer. Fig. 4 shows the absorption spectra of the reaction mixture obtained under standard conditions, in specially purified alcohol, with 0·1 mg. of androsterone (curve a) and of the blank, with reagents alone (curve b), comparison being made in each case with alcohol. The androsterone reaction shows a broad absorption band extending over the centre of the visible region, with a maximum at 5010 Å., whilst the reagents alone give low general absorption with a maximum at 4650 Å. Curve (c) shows the calculated difference between test and blank solutions. Reference to Fig. 5 shows that absorptiometric measurements with selective filters actually give a fairly faithful version of the net absorption spectrum after subtraction of the blank solution. The Ilford "spectrum-green" filter was selected for measurement of the absorption on the empirical ground that it gave the highest readings. The net absorption spectrum shows that any selective filter having maximum transmission somewhere between 5000 and 5400 Å. would be suitable for the purpose.

The reaction has been carried out generally under standard conditions, but in some cases using an earlier technique, with a number of methyleneketones, and the "absorptiometric spectra" have been measured with the Spekker absorptiometer and Ilford "spectrum" filters with the object of seeing whether compounds of different classes could be distinguished from one another. We are indebted to Messrs Ciba Ltd. for most of the androstane derivatives we have examined. The data obtained are in Table VI.

Influence of the position of the keto-group on the intensity and spectral characteristics of the colour. The first step in investigating the degree of specificity of the reaction with m-dinitrobenzene and alkali was to find whether it varied with the position of the keto-group in steroid compounds. Zimmermann [1936] had already shown that the approximate absorption spectrum given by testosterone was of a type different from that given by androsterone or oestrone, and Kaziro & Shimada [1937], using the Zimmermann technique, reported that the colour reaction was "positive" with 3-ketocholanic acid and "negative" with 6-, 7- and 12-ketocholanic acids.

The data we have obtained on various 3-, 6-, 17- and 20-ketones, given in detail in Table VI, show that the production of an intense, broad absorption band with a maximum in the green, is, among the steroid compounds, characteristic of the 17-ketones. In none of the groups of compounds is the type or intensity of the absorption much affected by distant substituents, and the spectra plotted in Fig. 5 may be taken as representative of their classes.

Among the *17-ketones*, we have investigated dehydroandrosterone in some detail with respect to factors influencing the intensity of the reaction on the same lines as androsterone. The effects of altering the concentration of the reagents and the time of development were the same as with androsterone. Dehydroandrosterone and oestrone gave calibration curves which followed closely that of androsterone. Androstan-17-one and $\Delta^{3,5}$-androstadien-17-one were very kindly given to us by Dr F. L. Warren of the Royal Cancer Hospital, and we take this opportunity of thanking him.

Table VI. "Absorptiometric spectra" of steroid and other methyleneketones and of neutral fractions of urine extracts with m-dinitrobenzene and KOH

Class and substance	Wt. (mg.)	Time of development	Absorptiometer readings (test − blank) and wavelength examined						
			E_v (max. 4500 Å.)	E_b (max. 4700 Å.)	E_{b-g} (max. 4900 Å.)	E_g (max. 5200 Å.)	E_{y-g} (max. 5600 Å.)	E_y (max. 5800 Å.)	E_o (max. 6100 Å.)
17-Ketones:									
Androsterone	0·1	1 hr.	0·21	0·26	0·305	0·385	0·35	0·31	0·18
	0·2	1 hr.	0·40	0·435	0·46	0·62	0·59	0·56	0·32
Dehydroandrosterone	0·2	1 hr.	0·30	0·34	0·42	0·58	0·475	0·44	0·265
Androstan-17-one	0·1	1 hr.	0·19	0·23	—	0·37	—	—	—
$\Delta^{3,5}$-Androstadien-17-one	0·2	1 hr.	0·365	0·41	0·47	0·62	0·555	0·53	0·135
	0·1	1 hr.	0·20	0·245	—	0·38	—	—	—
	0·2	1 hr.	0·43	0·45	0·48	0·67	0·61	0·61	0·355
Oestrone	0·1	1 hr.	0·16	—	—	0·42	—	—	—
	0·2	1 hr.	0·39	0·45	0·46	0·66	0·60	0·585	0·345
3-Ketones:									
Androstan-17-ol-3-one	0·2	20 min.	0·26	0·245	0·265	0·31	0·33	0·32	0·23
	0·2	1 hr.	0·225	0·18	0·17	0·21	0·19	0·19	0·135
Cholestanone	0·2	2 min.	0·18	0·195	0·205	0·26	0·285	0·265	0·175
	0·2	5 min.	0·26	0·23	0·24	0·295	0·33	0·325	—
	0·2	10 min.	0·235	0·21	0·22	0·28	0·31	0·31	0·22
	0·2	20 min.	0·20	0·175	0·175	0·23	0·25	0·265	0·185
	0·2	40 min.	0·16	0·115	—	0·15	0·145	0·15	—
	0·2	1 hr.	0·19	0·145	0·14	0·145	0·145	0·13	0·095
Δ^4-3-Ketones:									
Testosterone	0·1	1 hr.	0·18	0·15	—	0·155	0·15	0·165	—
	0·2	1 hr.	0·28	0·22	0·20	0·25	0·24	0·265	0·22
Cholestenone	0·1	10 min.	0·04	0·02	—	0·03	—	—	—
	0·1	20 min.	0·08	0·05	—	0·06	—	—	—
	0·1	30 min.	0·085	0·08	—	0·075	—	—	—
	0·1	1 hr.	—	—	—	0·135	—	—	—
	0·2	1 hr.	0·27	0·22	0·20	0·24	0·23	0·245	0·20

3:17-Diketone:									
Androstane-3:17-dione	0·1	1 hr.	—	—	0·33	0·41	0·37	—	—
	0·2	1 hr.	0·53	0·47	0·52	0·68	0·62	0·62	0·41
Δ⁴-3:17-Diketone:									
Δ⁴-Androstene-3:17-dione	0·1	1 hr.	—	—	—	0·42	—	—	—
	0·2	1 hr.	0·51	0·45	0·465	0·68	0·60	0·64	0·49
20-Ketones:									
epiallo-Pregnan-3-ol-20-one	0·1	1 hr.	—	—	—	0·045	—	—	—
	0·2	1 hr.	0·085	0·075	0·055	0·055	—	—	—
3:20-Diketone:									
Progesterone	0·1	1 hr.	0·20	0·19	—	0·19	—	—	—
	0·2	1 hr.	0·31	0·27	0·265	0·31	0·28	0·30	0·23
6-Ketones:									
Cholestan-6-one	0·2	1 hr.	—	—	—	0·01	—	—	—
Cholestane-3:6-dione	0·2	1 hr.	0·14	0·105	·09	0·11	0·10	0·115	0·09
Other compounds:									
cycloPentanone	0·058	5 min.	0·35	0·445	0·53	0·70	0·62	0·62	0·46
	0·058	10 min.	0·32	0·35	0·405	0·57	0·53	0·49	0·35
	0·058	20 min.	0·335	0·34	0·35	0·47	0·43	0·395	0·30
	0·058	30 min.	0·33	0·29	0·31	0·44	0·40	0·385	0·29
	0·058	1 hr.	0·38	0·32	0·30	0·40	0·36	0·33	0·25
cycloHexanone	0·067	1 hr.	0·35	0·22	0·175	0·19	0·14	0·12	0·18
Acetophenone	0·08	1 hr.	0·42	0·46	0·52	0·66	0·50	0·33	0·15
Acetone	0·04	1 hr.	0·295	0·29	0·27	0·29	0·22	0·20	0·15
Urine extracts (bulk collections)									
Female FU. 57.B	0·02 l.	1 hr.	0·18	0·19	0·205	0·27	0·24	0·21	0·135
FU. 62.B	0·01 l.	1 hr.	0·21	0·21	0·245	0·29	0·28	0·24	0·15
FU. 76.B	0·01 l.	1 hr.	0·30	0·30	0·33	0·43	0·405	0·36	0·225
Male MU. 38.B	0·011 l.	1 hr.	0·26	0·26	0·29	0·38	0·335	0·295	—
MU. 39.B	0·011 l.	1 hr.	0·23	0·225	0·265	0·32	0·30	0·26	0·165
MU. 75.B	0·01 l.	1 hr.	0·225	0·21	0·265	0·37	0·30	0·26	0·15

Saturated 3-ketones show a characteristic rapidity in development of the colour, which then fades, and after 1 hr. of development only a very low, general absorption is shown. The behaviour of cholestanone was investigated in detail, and the most intense colour, characterized by a band in the yellow and yellow-green, is given after only 5 min. development. This behaviour seems to be common to compounds having the group —CH₂—CO—CH₂—. *cyclo*Pentanone, for a specimen of which we are indebted to Prof. R. P. Linstead, gives a band with maximum in the green, which is developed rapidly.

Δ^4-*3-ketones*, in contrast to the saturated 3-ketones, require a long time of development, and, in the case of cholestenone, some detailed figures for which are given in Table VI, it seems that development of the maximum colour is not complete after 1 hr. All the Δ^4-3-ketones show a characteristic peak in the yellow, in addition to one in the green, and this is visible even when the powerfully chromogenic 17-keto-group is also present, as in Δ^4-androstene-3:17-dione (cf. Fig. 5).

The *20-keto-group* gives a very low, but measurable general absorption. In combination with a Δ^4-3-keto-group it causes a small general rise in the absorption.

The *non-steroid* compounds were measured in amounts which were the molar equivalents of 0·2 mg. of androsterone. Acetone shows the rapid development of colour characteristic of dimethyleneketones. Acetophenone is noteworthy for the resemblance of the curve to that of 17-ketosteroids, except for considerably lower absorption in the yellow and yellow-green. This resemblance, in spite of a considerable difference in structure, demonstrates with what caution absorption spectra of this colour reaction should be accepted as indicative of structure without confirmatory evidence.

Absorption spectra of the colour reaction with urine extracts. The absorptiometer measurements obtained with extracts from bulk collections of male and female urines are given in Table VI and plotted in Fig. 6 where they are compared with the absorptiometric spectrum of 0·1 mg. of androsterone. It is evident that there is no characteristic difference between extracts from either male or female urine and a typical 17-ketosteroid beyond the minor one of having a value for E_v which is relatively higher and of the same order as E_b. However, in certain abnormal cases there are major differences in the absorption in the shorter wavelengths. We have, in practice, made measurements of E_r and E_v as a routine, in addition to E_g. Certain urines yield extracts which give at $E_g = 0·37$ values of E_v or E_b or both which are of the same order as E_g ($E_v/E_g > 0·8$). It is clear that these contain chromogenic compounds not present in appreciable amount in normal urine, and, since the value of E_g is liable to be affected by absorption in the adjacent regions of the spectrum, there must be some doubt whether such extracts can fairly be compared with others on a scale of values of E_g. The ratio E_v/E_g tends to be high when the chromogen content is low, and is probably then an indication of the presence of a substance giving rise to general absorption. There is also qualitative evidence, from the rate of development of colour with urine extracts, that substances like saturated 3-ketosteroids are present in small amount. Although in certain cases an overestimate of the biological activity was made when the value of E_v/E_g was high, in other cases, in which, presumably, the urine component giving absorption in the violet did not affect E_g, the relation was normal, and no general rule can be laid down except to regard with suspicion an extract with a high value of E_v/E_g. In the following treatment of the correlation of colorimetry and biological assay no selection of urine extracts in respect of values of E_v/E_g has been made. In two

cases the abnormal colour was traced to contamination resulting from extraction of vulcanite stoppers by toluene used to preserve the urine. Naturally, such contamination must be avoided in collecting specimens intended for colorimetric assay.
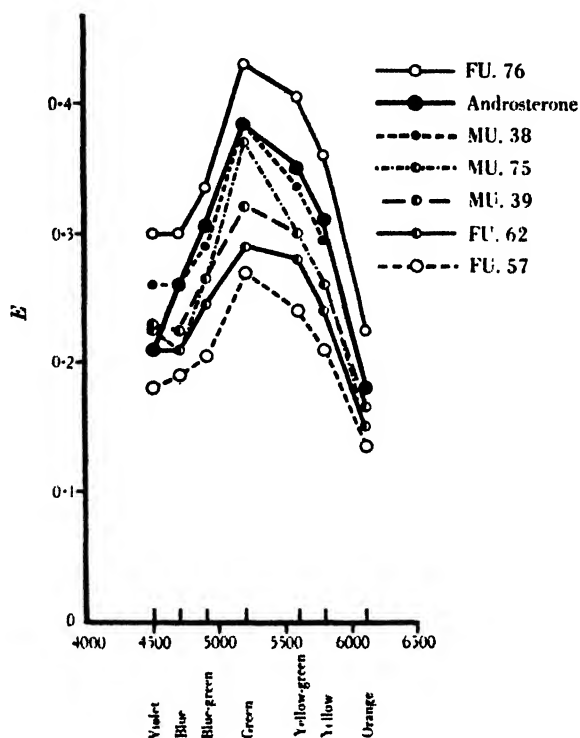
Fig. 6. "Absorptiometric spectra" of the reaction of extracts of men's (MU.) and women's (FU.) urine + m-$C_6H_4(NO_2)_2$ + KOH compared with 0·1 mg. of androsterone. (FU. 57 equiv. 0·02 l.; other urine extracts equiv. 0·01 l.)

(4) Correlation of colorimetry and biological assay

Method of biological assay. The urine extracts in oily solution have been assayed for androgenic activity by a capon comb-growth method, described in detail elsewhere [Emmens, 1938]. A dose/response curve was constructed by giving injections of international standard androsterone, and checked regularly by at least one control group of 5 capons. Androgenic activity is expressed as being equivalent to so many international units of androsterone per litre (1 I.U. = 100 μg.), and the estimates based on groups of five birds. In four instances an extract has been assayed by the direct inunction of an oily solution on the comb, and compared with androsterone similarly administered. These four estimates fall well within the range of the others, there being therefore no reason to exclude them from the calculations, despite the difference of technique. Their influence on any conclusions drawn from the data is in any case negligible.

Urines investigated. In the course of the scheme of research of which this investigation forms a part, a variety of urines has been extracted and examined, including bulk collections from groups of normal men or women, a small number from men and women in hospital with no obvious sexual dysfunction, and urine

from eunuchs, eunuchoids, ovariectomized women, cases of Addison's disease and hirsutism in females. For the purpose of considering whether colorimetric and biological assays could be correlated, all these have been lumped together; only cases in which hormone-producing tumours have been proved to be present have been excluded. It may therefore be considered that the hypothesis of correlatability is put to a severe test. The possibility of distinguishing between these classes of patients will not be discussed here.

Statistical treatment of the results

Fig. 7 is a scatter-diagram showing the correlation between the colorimetric and biological assay figures for 59 urine extracts, which are given in Table VII. These were obtained from collections from the following: normal men, 4 groups
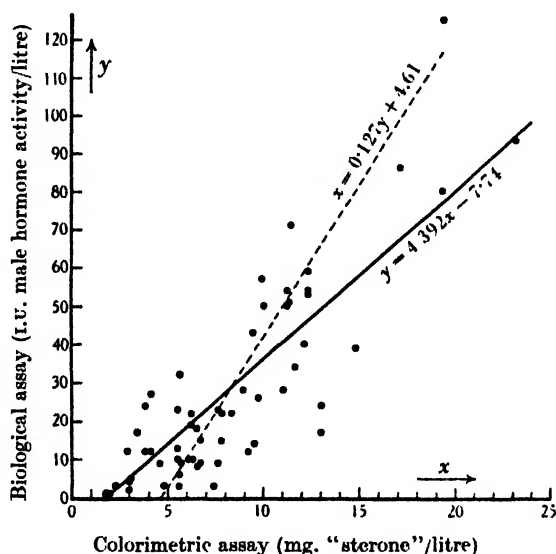
Fig. 7. Correlation of capon comb-growth assay and colorimetric assay of 59 urine extracts.

and 7 individuals; normal women, 4 groups and 6 individuals; eunuchs, 8 (9 samples); ovariectomized women, 8; eunuchoids, 2; hirsute women, 8; cases of Addison's disease, 7 (6 samples from 4 women; 3 men); carcinoma of cervix, 2. To provide comparable figures for calculation, all quantities have been expressed as per litre, though the quantities per day are the significant ones for individual patients and have been measured in most cases. A correlation coefficient has been calculated by grouping the data with units equal to 10 I.U. and 2 mg. "sterone", this coefficient being 0·745. The two regression lines, $y = 4·392x - 7·74$ and $x = 0·127y + 4·61$ express, respectively, the regression of content of international units by capon assay (y) on "sterone" content (x), and that of "sterone" content on international unit content. The appearance of a better "fit" given by the first of these lines, and the choice of it as a basis for discussion of the meaning of the results depends on the relative errors of the colorimetric and biological tests.

It is known that the error of the capon comb-growth test may be large, but a satisfactory estimate of the average value of this error for a large number of tests is almost impossible to obtain since it depends on both the age of the

Table VII. *Colorimetric and biological assays of urine extracts*

Type and ref. No.		mg. "sterone"/l.	i.u./l.	Type and ref. No.		mg. "sterone"/l.	i.u./l.
(i)	1	1·8	1	(f)	16	7·6	9
(j)	11	2·0	1*	(d)	21	7·6	23
(i)	3	2·3	3	(b)	84	7·8	15
(j)	6	2·9	12	(e)	72	7·8	22
(e)	64	3·0	2*	(c) FU.	62	8·3	22
(e)	77	3·0	4	(a) MU.	75	8·9	28
(f)	14	3·1	5	(f)	15	9·2	12
(j)	8	3·4	17	(b)	31	9·4	43
(c) FU.	57	3·8	12	(h)	25	9·5	14
(d)	51	3·8	24	(d)	31	9·7	26
(b)	61	4·1	27	(a)	44	9·9	57
(e)	68	4·2	12	(f)	10	10·0	50
(i)	7	4·6	9	(c) FU.	76	11·0	28
(h)	21	4·8	3	(k)	50	11·2	50
(h)	27	5·5	10*	(a)	65	11·2	54
(f)	9	5·5	13	(b)	2	11·3	51
(i)	9	5·5	23	(d)	11	11·4	71
(e)	98	5·6	3	(a) MU.	38	11·6	34
(e)	86	5·6	6	(h)	26	12·1	40
(b)	11	5·6	32	(b)	21	12·3	53
(e)	104	5·7	9	(g)	40	12·3	54
(d)	81	6·1	10	(g)	33	12·3	59
(f)	12	6·2	19	(h)	31	13·0	17
(i)	4	6·2	22	(k)	51	13·0	24
(e)	38	6·3	10	(d)	121	14·8	39
(f)	11	6·5	18	(h)	38	17·1	86
(e)	65	6·55	8	(h)	34	19·3	80*
(i)	5	6·7	9	(f)	13	19·4	125
(b)	81	6·7	15	(h)	24	23·2	93
(e)	73	7·4	3				

(a) Bulk collection, normal men. (e) Eunuchs. (i) Addison's disease, women.
(b) Normal men. (f) Ovariectomized women. (j) Addison's disease, men.
(c) Bulk collection, normal women. (g) Eunuchoids. (k) Carcinoma of cervix.
(d) Normal women. (h) Hirsute women.

* Capon assay by inunction.

birds and the amount of growth stimulated [Emmens, 1938]. However, 20 control groups receiving androsterone for a period during which capon variability was on the high side, and during which approximately one half of the estimates used here were made, yield a correlation of 0·862 between comb-growth and the amount of androsterone injected. The correlation for the whole period is certainly not less than this, and probably as high as 0·9. The z test [Fisher, 1932] reveals that a correlation of 0·745 derived from 59 pairs (the number of extracts assayed) is not significantly different from one of 0·862 derived from 20 pairs. One can conclude therefore that the correlation between biological and colorimetric assays is not much less than that between biological assay and the true amount of androgenic material present, even when the latter is pure androsterone.

The correlation between values of E_g and the amount of androsterone present cannot be accurately expressed by a correlation coefficient, since the relationship is not linear. Fig. 1 demonstrates that the colorimetric determination of androsterone is very accurate, although the accuracy with which urine extracts have been assayed is appreciably less than that indicated in Fig. 1 and the calculation on p. 1315 suggests. There remains no doubt, however, that the greater part of the departure of the correlation between colorimetric and biological assays from unity is due to the errors of biological assay. The small contribution of the errors of colorimetric assay to this departure probably does

not account completely for the remainder, since a perfect fit of the biological and colorimetric estimates could be expected only on uniform material. Thus, if it were assumed that androsterone and dehydroandrosterone were the active substances, differences from sample to sample in the ratio of these two compounds would reduce the correlation of the two estimates, unless balanced by appropriate variations in the content of biologically inactive chromogens. In view of the fact that a given colorimetric reading may be obtained from a series of mixtures of different active and inactive chromogens, each giving a different result when tested biologically, the degree of correlation found is high.

Chemical evidence

Certain preliminary work has been carried out on the analysis of urine extracts into their constituents with a view to the complete identification of the latter; results have been obtained which, subject to the reservations necessitated by the inherent error of capon assay, and the rather unquantitative character of the methods, indicate the association of biological activity with the chromogenic, ketonic fraction. Separation of the ketonic fraction as water-soluble compounds by treatment with Girard's reagent T (trimethylammonium acethydrazide chloride) [Girard & Sandulesco, 1936] gives results such as the following:

450 mg. of extract MU. 75[1] equivalent to 10·25 l. of urine, assayed at 8·9 mg. of "sterone" per litre by colorimetry, or 28 international units of androgenic activity per litre by capon comb growth, yielded, after extraction with methanol and two treatments with Girard's reagent, (a) a high-ketone fraction, wt. 86 mg., "sterone" content 6·8 mg. per litre, androgen activity 45 i.u. per litre, (b) two low-ketone fractions, one, weight 12 mg., "sterone" 0·19 mg. per litre, and the other, weight 225 mg., with a "sterone" content of 0·37 mg. per litre (but with a high value of E_v), and androgen activity less than 2 i.u. per litre. This shows clearly that the androgenic compounds are confined to the ketonic fraction.

DISCUSSION

The use of "male hormone assay" of urine extracts by biological methods as a method of diagnosis of the physiological condition of a patient entails the assumption, originally based, perhaps, on a presumed specificity of male hormone activity, that the circulating hormones, although they may be destroyed or transformed in the course of excretion, will yet give a product whose activity will be proportional to the physiological activity of the original compounds present in the human body. An incidental assumption is made in supposing that the usual (and only practical) method of assessing the biological activity by the comb-growth of capons gives a measure of the activity in the human body. The factual support for the main assumption is slender, owing to the immense difficulties of chemical analysis of extracts of tissue, blood and excreta. The only compounds with marked androgenic activity so far isolated from the organism are testosterone from the testis, and Δ^4-androstene-3:11:17-trione from adrenal cortical tissue. The only active compounds which have been isolated from normal urine are androsterone and *trans*dehydroandrosterone, or Δ^4-androsten-3-*trans*-ol-17-one, and the amounts identified account for only a fraction of the biological activity. Urines from adrenal tumour cases have

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[1] We are indebted to Messrs Boots Pure Drug Co. for this extract made at Nottingham from a 250 l. bulk collection of men's urine. Other data from this extract have been used in Tables VI and VII.

yielded $\Delta^{5:7}$-androstadien-17-one [Burrows *et al.* 1937], and *trans*dehydroandrosterone [Callow, 1936, 2]; in the last case the amount of the compound isolated accounted for 70% of the biological activity. A related, inactive compound, *epi*aetiocholanediol, has been isolated in very small amount from normal urine extract [Butenandt *et al.* 1937]. The genetic relationship between secretion and excretion is thus far from clear with respect to chemical structure. The investigation of the relationship with respect to biological activity is as yet hardly begun, though from the work of Kochakian [1937] and from work carried out in this laboratory in collaboration with clinicians, a preliminary account of which has been given in a lecture by one of us [Callow, 1938], it is already evident that the recovery of androgenic activity from urine extracts is only of the order of 5%, as a maximum, of that administered by injection. Here again, the scale of comparison is comb growth in the capon, although it is known that the relative activities of androgens, which vary even in capons according to the mode of administration, are different in laboratory mammals, and, presumably, in man. Further, the biological assay is of a mixture of substances, and, of two known constituents of this mixture, one has about three times the activity of the other.

It seems, therefore, that an examination of urine extracts by a chemical test, to which the known active urine constituents and closely related steroids respond, will entail no more dangerous assumptions than examination by biological assay.

The first result of this work is that of increasing the accuracy and reproducibility of a method of determining 17-ketosteroids. It has then been shown that a greater degree of specificity can be introduced into this determination by rough measurements of the absorption spectra. A claim for complete specificity would be untenable unless a more exact examination of a far wider range of compounds had been made. The hypothesis that the method gives a measure of the 17-ketosteroids in urine extracts is justified and supported by three considerations. First, the method of obtaining the extracts is such that neutral, non-volatile compounds of high molecular weight are separated. Secondly, after chemical fractionation of the extract, the chromogenic activity is concentrated in the ketonic fraction. Thirdly, the absorption spectra of the coloured compounds formed by this material with *m*-dinitrobenzene in alkali closely resemble those of the compounds formed by pure 17-ketosteroids.

The further hypothesis that a determination of 17-ketosteroids made in this way gives a measure of the androgenic activity is suggested in the first place by the knowledge that two of the compounds to which urine extracts owe their androgenic activity are actually 17-ketosteroids. Secondly, there is the inherent likelihood that 17-ketosteroids will all possess androgenic activity. Confirmation is afforded by the degree of correlation between colorimetric and capon assays. When it is considered that the urine extracts are certainly a complex mixture of compounds with both androgenic and chromogenic properties, and that the first step in obtaining them is a vigorous acid hydrolysis involving both liberation and destruction, which may be selective, under conditions difficult to control, and, further, that an absorption spectrum is a physical property particularly sensitive to impurities, it might well be expected that even if the natural urine contained a simple mixture of androgens in constant proportions, the inherent correlation between colorimetric and biological assays would be blurred beyond recognition. It has been shown that a significant degree of correlation does exist. Thus, if it is a question of deciding, on theoretical grounds of chemical metabolism or physiological relationship, whether chemical or biological assay is the better, we can make the best of both worlds, and employ either. Neither

form of assay is specific, but the chemical one is more susceptible to investigation and improvement in this respect, and has the added advantage of rapidity and smaller inherent error: it would seem at least as likely to give diagnostically significant results.

The contributions of this investigation to the question of the exact composition of the urinary excretory transformation products of male hormones are an improved chemical test for a type of these products, but not for any individual compound, and an indication of a relationship of chromogenic to biological activity concordant with a simple working hypothesis without giving it preferential support. The relationship $y = 4\cdot392x - 7\cdot74$, which gives the most likely estimate of the biological assay equivalent to a given colorimetric reading, is intermediate between that which would be given by androsterone and dehydroandrosterone. In practice pure androsterone would give an equation close to $y = 10x$, and pure dehydroandrosterone one approximating to $y = 3\cdot3x$, since the activity of the latter on capons is about one-third of that of androsterone. It might be assumed that the androgenic and chromogenic material in urine extracts is a mixture of androsterone and dehydroandrosterone, the two substances which have actually been isolated from normal male urine. an assumption which has been made, on other grounds, by Dingemanse & Laqueur [1938]. However, the intermediate position of the experimentally determined line is not in itself proof of the presence of a mixture of androsterone and dehydroandrosterone in the average urine dealt with. It could result from the presence of androsterone and biologically inactive chromogen only. The presence of some inactive chromogen is indicated by the constant $7\cdot74$, which is a measure of the amount found when little active substance is present, whilst there may be more inactive chromogen in urines with higher biological activity. Moreover, there is a wide discrepancy between the amounts of androsterone and dehydroandrosterone recorded as actually isolated (about 1 mg. per litre of each) and the amounts indicated by biological assay. From the evidence now presented, no more definite conclusion can be drawn, than that the content of biologically active material is proportional to the content of 17-ketosteroid compounds.

SUMMARY

1. A description is given of a modified and improved method of determining androsterone and other 17-ketosteroids by the colour reaction with m-dinitrobenzene and potassium hydroxide in alcoholic solution.

2. The sources of error and the factors influencing this determination have been investigated.

3. Examination of the behaviour of other methyleneketones has shown that the absorption spectrum of the coloured solution produced and the rapidity of development of the colour vary according to the structure of the compound.

4. The neutral fractions of extracts of hydrolysed urine give the colour reaction characteristic of 17-ketosteroids, although with some indication of the presence of other chromogenic compounds. Further, biological activity is associated with the ketonic fraction. These facts justify the use of the reaction for estimating the excretory transformation products of male hormones.

5. A comparison of the chemical with the biological method of determining the excretory products by their activity in the capon comb-growth test has been made with 59 urines from a variety of sources. A significant degree of correlation is found between the colorimetric assay expressed in chromogenic equivalents

of androsterone ("sterone") per litre of urine and the biological assay expressed in international units of male hormone activity per litre.

6. It is concluded that colorimetric assay can replace biological assay provided that due regard is paid to the occasional presence of interfering substances, which may be revealed when the approximate absorption spectrum is examined.

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CLXXVII. THE EFFECTS OF PANCREATIC EXTRACTS ON FAT DEPOSITION IN THE DIETARY FATTY LIVER

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ALLAN *et al.* [1924] reported that depancreatized dogs receiving adequate amounts of insulin and maintained on a diet of lean meat, sucrose and bone ash did not survive for periods of more than a few months. They also observed that the symptoms of failure of liver function due to fat infiltration of the liver found in such animals, could be prevented by adding raw pancreas to the diet. They suggested, therefore, that the pancreas might possibly produce an internal secretion necessary for the physiological integrity of the liver. The subsequent discoveries of Hershey [1930] that lecithin could successfully replace raw pancreas in the diet of depancreatized dogs, and of Best & Huntsman [1932] that choline and betaine could prevent and cure fatty infiltration in the livers of rats fed on high fat diets have been extended in many directions. So far as we are aware, however, no proof has yet been advanced that the active agent of the pancreas is, in fact, choline, and particular interest therefore attaches to the claims of Dragstedt and his colleagues [Dragstedt *et al.* 1936, 1, 2] that raw pancreas contains an active substance other than choline and that this substance, to which they give the name "lipocaic" may be concentrated in dilute alcoholic extracts of the tissue.

The work described in this paper was undertaken in an attempt to extend these findings to the dietary fatty liver of the rat, which lends itself to more accurate experimentation than that of the depancreatized dog.

While these experiments were in progress, other workers reported the results of similar studies [Chaikoff & Kaplan, 1937; Kaplan & Chaikoff, 1937; MacKay, 1937; Shapiro & Wertheimer, 1937; Aylward & Holt, 1937; MacKay & Barnes, 1938; Best & Ridout, 1938]. Some of these results throw doubt on the existence of a pancreatic factor other than choline influencing fat deposition in the liver, while in some cases the conclusions drawn appear to us to be based on inadequate data. These points are discussed later in this paper.

The general plan of the experiments has been to feed control groups of rats on diets which cause fatty livers. At the same time other groups have received the same diet with the addition of varying amounts either of pancreatic extract or of choline, in order to assess in terms of choline the activity of pancreatic extract in preventing fat deposition. It is difficult to strike the correct range of choline dosages in attempting to assay an unknown substance and many more groups of animals were used than are recorded in the tables which follow. Tables II–V contain, therefore, only the results of the 42 groups which are relevant to the discussion, either because they provide the actual results on which the effect of the pancreatic extract has been assessed, or alternatively because it is important in forming a decision as to the activity of the pancreatic

extracts, that the effects of increasing amounts of choline should be recorded for comparison, in order that the accuracy of the assay may be suitably appraised.

Because of the differences of opinion evident from the publications referred to above, it appeared to us necessary to obtain a considerable body of evidence. Accordingly six experiments were carried out, three on the "cholesterol", and three on the "fat" fatty liver.

EXPERIMENTAL

Preparation of the extracts

Fresh ox pancreas obtained from the slaughter house was freed from extraneous connective tissue and fat.

Dragstedt *et al.* [1936, 2] extracted pancreas with saline and with alcohol of various dilutions and found that saline or 40 % alcohol gave more active extracts than 70 % alcohol. In the experiments now reported the method of extraction used has followed closely that of these workers except in two particulars: (*a*) in the initial extraction of each batch alcohol was added so that, allowing for the water content of the pancreas, the final concentration was 40 %, all the succeeding extractions being carried out with 40 % alcohol save in Exp. 2: (*b*) instead of evaporating the extracts in open dishes and extracting the residual solid with ether, alcohol and water were removed *in vacuo* and extraction carried out by shaking with ether in a funnel before the material became pasty. In all the experiments the extracts were made up to a definite volume, and stored in the refrigerator: aliquots providing sufficient of the extracts for incorporation in 3-day portions of diet for all groups receiving pancreatic extract were withdrawn into test tubes, pasteurized, sealed and stored in the refrigerator for use as required.

Exp. 1. 1·2 kg. of minced pancreas were left overnight with 2·4 l. of 60 % alcohol, strained through muslin and twice re-extracted with 40 % alcohol (2·4, 2·4 l.); the residue was treated with ether (2·5, 2·0, 2·0 l.). The first "ether" extract which contained much alcohol and water was added to the 40 % alcohol extracts and the whole evaporated *in vacuo* at 60°. Fat was removed from the concentrate by shaking with the second ethereal extract of the protein residue and four times subsequently with fresh ether (1 l.); alcohol and ether were then removed from the aqueous suspension *in vacuo* and the volume made up to 1·2 l. with water.

Exp. 2. 5 kg. minced pancreas were treated three times with 40 % alcohol as in Exp. 1: the protein residue was then extracted with 95 % alcohol (2. 3 l.), the extracts evaporated *in vacuo* and added to the previous extracts. Much of the fat thus added to the 40 % alcohol extracts was removed by ether, but the high choline content (Table 1) indicates that some phosphatide was probably left in the aqueous layer.

Exp. 3. 1·2 kg. of minced tissue worked up as in Exp. 1.

Exp. 4. The extract prepared for Exp. 3 was used. In addition, the effect of precipitation with trichloroacetic acid was investigated. 40 ml. of the extract of Exp. 3, equivalent to 96 g. of pancreas, were treated with an equal volume of 10 % trichloroacetic acid, and the precipitate filtered off. The filtrate was extracted six times with ether to remove trichloroacetic acid, evaporated and then made up to 96 ml. The precipitate represented 6·4 % of the solids taken, and the filtrate 77·4 %.

Exp. 5. 250 g. of minced tissue extracted as in Exp. 1.

Exp. 6. 2 kg. of minced tissue extracted as in Exp. 1; evaporation of the extracts at a bath temperature of 30–40° was continued to dryness and the solid extracted with 500 ml. ether. The residual solid after removal of ether was dissolved in 500 ml. water.

The choline content of each extract was determined by biological assay on the isolated rabbit intestine after hydrolysis and acetylation. It was thus

possible to decide on the amount of choline to be given to the groups receiving choline, in order to assess the non-choline activity of the pancreatic extracts. In addition, the total solids present in each extract, save in that of Exp. 5, were obtained by drying to constant weight at 105°, and the ash content determined. In Exps. 1, 2 and 6 estimations of total N and in the former two of non-protein-N on the filtrate obtained after precipitation with sodium tungstate and H_2SO_4 were made. The results of these analyses are recorded in Table I.

Table I. *The solids of the pancreatic extracts*

Exp.	Yield % fresh pancreas	% of total solids			
		Choline	Total N	N.P.N.	Ash
1	5·5	1·0	10·9	8·0	13·5
2	12·7	2·45	11·8	10·8	8·7
3	10·45	1·64	—	—	10·1
4	10·45	1·64	..	--	10·1
Trichloroacetic acid filtrate	8·08	2·12	—	—	10·8
5	(1·8 mg. choline/g. pancreas)	--	—	—	—
6	4·64	3·3	9·5	..	16·0

Animal feeding

In Exps. 1, 2 and 6 diets which result in "cholesterol" fatty livers, and in Exps. 3, 4 and 5 diets producing "fat" fatty livers were employed. The diets and the periods of feeding were as follows.

Exp. 1. Caseinogen (alcohol-extracted) 5, cholesterol 2, beef dripping 29, cod liver oil 1, glucose 53, marmite 5 and salts 5 parts; 21 days.

Exp. 2. As in Exp. 1 for 15 days.

Exp. 3. Caseinogen 5, beef dripping 39, cod liver oil 1, glucose 50, salts 5 parts with 10 μg. aneurin per rat per day; 16 days.

Exp. 4. As in Exp. 3 save that the caseinogen was increased to 8 parts at the expense of 3 parts of glucose, while after 7 days it was replaced by commercial egg albumin; 16 days.

Exp. 5. As for the first 7 days of Exp. 4 save that the daily intake of aneurin was increased to 12 μg. and each rat also received 24 μg. lactoflavin; 15 days.

Exp. 6. Commercial egg albumin 8, beef dripping 39, cod liver oil 1, glucose 45, cholesterol 2, salts 5 parts; each rat received 25 μg. aneurin daily; 19 days.

The supplements of pancreatic extract and of choline as chloride were dissolved in the same volumes of water and incorporated in 3-day portions of the dry diets; for the control diets an equal volume of water was used.

At the beginning of the experiments, all the groups except two control groups contained 10 animals (5 ♂, 5 ♀), suitably chosen so that animals of the same weight and sex appeared in each group. At the conclusion of the feeding period, animals which showed a weight loss greater than 20 % or any other abnormality were rejected. As a result, when the animals were killed 35 of the groups each contained either 9 or 10 animals, groups 14, 21 and 22 contained 8, and groups 28 and 38 only 7; two control groups 18 and 27, which began as groups each of 20 animals, were finally reduced to 18 and 19 respectively.

Analysis of livers

The methods of investigating the livers and the analysis of the lipoids were as follows. In Exps. 1, 2 and 6 in which the "cholesterol" fatty liver was employed, the total ethereal extract of the pooled livers of each group of animals was prepared. In two of these experiments, it was completely analysed for

phosphatide, cholesterol, cholesteryl esters and glyceride. Since only the glyceride fraction is pertinent to the discussion of the results, the amounts of the total lipoids and glyceride only are recorded (Tables 11 and V). In Exp. 2 a detailed analysis was not made, fatty acids and unsaponifiable matter only being determined after saponification. In this experiment the total fatty acids and the total lipoids only are recorded (Table III). Previous experience has shown that for all practical purposes differences in the total fatty acid contents of the livers of the different groups may be regarded as differences in glyceride. In Exps. 3–5 in which the "fat" fatty liver was investigated the livers of all the animals were individually analysed by the saponification method for their content of fatty acids and unsaponifiable matter. The "fat" contents of the different groups of these three experiments recorded in Table IV are thus the mean values of the fatty acids and unsaponifiable matter of the individual animals of each group.

Food intake

In Exp. 1 the daily food intake of all the animals was measured, and in Table II the amounts of pancreatic extract or of choline recorded are the actual amounts ingested by the different groups. In Exps. 2–6 the daily food consumption was not determined and the amounts of pancreatic extract or choline recorded in Tables III–V have been calculated on the basis that each rat consumed 8 g. of diet per day. Much previous experience suggests that this is a reasonable figure for purposes of calculation for animals of the weight used: further, since the average weight losses in the various groups of each experiment were similar, no serious error will be introduced in using such calculated values for comparative purposes, as will be shown later. In the tables the amount of pancreatic extract is recorded as the daily intake of the weight of pancreas from which it was derived ("pancreas equivalent"), and also as the daily intake of its contained solids ("actual weight"). The "extra choline intake" represents the choline additional to that present in the basal diet received by the different groups either in the pancreatic extract or as administered choline chloride.

RESULTS

When a liver becomes fatty, the percentage of fat in it increases and at the same time its size increases. It is thus possible to discuss the results from both these points of view, but since the calculated weight of "fat" in the liver of the 100 g. rat reflects both these changes, it gives a more complete picture of the results. Accordingly, the results will be discussed only on the basis of the weight of the constituents in the liver of the 100 g. rat, although both the liver size and the percentages of "fat" are recorded in Tables 11–V.

In Table II the extra choline intakes of groups 2 and 3 which received pancreatic extract, appear as negative values, because the food consumption of these groups was less than that of the others, the basal diets in both Exps. 1 and 2 being relatively rich in choline (32·5 mg. per 100 g.), since they contained marmite.

The results of groups 2 and 3 show that the administration of the extract derived from 0·44 and 0·08 g. of pancreas has lowered the amount of glyceride in the livers from the control figure of 1·96 g. to 0·88 and 1·11 g. respectively. These marked decreases show clearly the considerable effect of the pancreatic extract in preventing fat deposition in the liver. Further, this effect is not due to choline, for the choline intakes of these two groups were actually less than

Table II. *Exp. 1. Comparative activities of choline and pancreatic extracts on glyceride deposition in the "cholesterol" fatty liver*

Group	Supplement administered	Average initial body wt. g.	Wt. loss % initial body wt.	Food intake g.	Extract administered		Extra choline intake/rat/day mg.	Liver % final body wt.	Total lipoid		Glyceride	
					Actual wt./rat/day mg.	Pancreas equivalent g.			% fresh liver	In liver of 100 g. rat g.	% fresh liver	In liver of 100 g. rat g.
1	—	180	6	9·0	—	—	—	6·3	37·3	2·34	31·3	1·96
2	Pancreatic extract	176	7	7·4	24·4	0·44	−0·3	4·7	24·6	1·14	19·0	0·88
3	Pancreatic extract	165	4	8·0	4·4	0·08	−0·3	5·0	29·2	1·46	22·1	1·11
4	Choline	167	2	9·1	—	—	9·2	4·0	13·9	0·54	7·4	0·29
5	Choline	167	2	8·5	—	—	4·1	4·6	20·3	0·93	13·7	0·63

Table III. *Exp. 2. Comparative activities of choline and pancreatic extracts on total fatty acid content of "cholesterol" fatty liver*

Group	Supplement administered	Average initial body wt. g.	Wt. loss % initial body wt.	Extract administered		Extra choline intake/rat/day mg.	Liver % final body wt.	Total lipoid		Fatty acids	
				Actual wt./rat/day mg.	Pancreas equivalent g.			% fresh liver	In liver of 100 g. rat g.	% fresh liver	In liver of 100 g. rat g.
6	—	143	0	—	—	—	5·9	30·9	1·83	25·1	1·48
7	Pancreatic extract	139	4	93	0·74	2·3	4·3	18·6	0·80	13·0	0·56
8	Pancreatic extract	161	4	46	0·37	1·2	4·7	22·8	1·07	17·3	0·81
9	Pancreatic extract	146	5	24	0·19	0·6	5·2	30·3	1·58	24·1	1·26
10	Pancreatic extract	154	6	9	0·07	0·2	5·5	34·4	1·89	28·8	1·58
11	Pancreatic extract	149	8	1	0·008	0·02	5·0	32·5	1·64	26·2	1·32
12	Choline	153	4	—	—	5·5	4·3	18·7	0·80	13·7	0·58
13	Choline	161	13	—	—	3·9	4·0	18·8	0·75	13·6	0·54
14	Choline	156	9	—	—	3·1	4·1	15·2	0·62	10·6	0·53
15	Choline	166	7	—	—	2·3	4·5	21·6	0·96	16·7	0·74
16	Choline	162	7	—	—	1·6	4·6	24·8	1·15	19·6	0·91
17	Choline	158	5	—	—	0·8	5·7	32·2	1·83	26·8	1·52

that of the control group. Since the greater decrease in group 3, 1·08 g., is less than the 1·33 g. decrease caused by 4·1 mg. choline in group 5, it is impossible accurately to assess the activity of the extract in terms of choline. If the arbitrary assumption be made that the preventive action of choline is linear over this range of liver fat percentages, then the extract from 0·44 g. pancreas has been as effective as $\frac{108}{133} \times 4 \cdot 1$ mg. choline. The pancreatic extract thus has an activity equivalent to 757 mg. choline per 100 g. pancreas, whereas its contained choline represents 55 mg. per 100 g. i.e. its activity is about 14 times that of its contained choline. While the assumption as to the linear nature of the effect is probably not strictly accurate, the results of this experiment appear conclusive in showing the presence in the pancreatic extract of an active substance other than choline. It is to be mentioned also that if the decrease in group 3 were equated with that of group 5 in the same manner as was done for group 2, the activity of the pancreatic extract would have appeared considerably greater even than that found above.

The results in Table III illustrate the limitations of the experimental method, and the caution which is necessary in interpreting the results. The livers of the control group contained 1·48 g. fatty acid: this decreases to 0·56 g. with the highest dose of extract (pancreas equivalent 0·74 g.) and with succeeding doses the amount increases until in group 10 with a pancreas equivalent of 0·07 g., it is 1·58 g., a figure very similar to that of the control group. In the last of the pancreatic groups, however, the amount falls to 1·32 g. yet clearly it should be identical with that of the control group, since the amount of the pancreas equivalent was negligibly small in this group. This illustrates the variations which are to be encountered even when groups of 10 animals are used and emphasizes the unreliability of attempts to equate the effect of any particular dosage of pancreatic extract unless it has a pronounced effect in decreasing the amount of liver fat.

The choline groups, 12–17, illustrate another difficulty, namely that it is not possible to demonstrate clearly changes caused by small successive decreases in the amount of choline administered, unless the doses employed are near the limiting effective amounts of 2–3 mg. Thus each animal of groups 12, 13 and 14 received daily 5·5, 3·9 and 3·1 mg. choline respectively: yet the amounts of fat in the livers of these three groups, 0·58, 0·54 and 0·53 g. are the same. It is thus impossible to differentiate between the effects of 3·1, 3·9 and 5·5 mg. choline. The decreasing effects of amounts of choline lower than these appear, however, in groups 15, 16 and 17, in the last of which with an intake of only 0·8 mg. the amount of fatty acids in the liver, 1·52 g., has returned to the control figure 1·48 g.

The results of pancreatic group 7, therefore, could be equated with those of either of the choline groups 12, 13 or 14. In order to obtain the minimum figure for the activity of the pancreatic extract it is considered wise to equate group 7 with group 14 although it must be emphasized that the effect of the pancreas may be underestimated by so doing. If this be done the extract from 0·74 g. pancreas and containing 2·3 mg. choline has an action equal to 3·1 mg. choline. A second value may be obtained from the results of group 8, where the liver fat is 0·81 g., which lie between those of groups 15 and 16 in which the amounts are 0·74 and 0·91 g. respectively. If an intermediate value be taken, a pancreas equivalent of 0·37 g. and containing 1·2 mg. choline is equivalent in action to 2·0 mg. choline. From the results of groups 7 and 8, therefore, the pancreatic extract in this experiment had an action equal to that of 419 and 541 mg.

Table IV. *Comparative activities of choline and pancreatic extracts on total "fat" content of "fat" fatty liver*

Group	Supplement administered	Average initial body wt. g.	Wt. loss % initial body wt.	Extract administered Actual wt./rat day mg.	Pancreas equivalent g.	Extra choline intake/rat/day mg.	Liver % final body wt.	"Fat" % fresh liver	"Fat" in liver of 100 g. rat g.
				Exp. 3					
18	—	154	12				4·7	21·7	1·07
19	Pancreatic extract	156	12	100	0·96	1·6	3·7	9·2	0·34
20	Pancreatic extract	155	9	80	0·77	1·3	3·8	10·9	0·42
21	Pancreatic extract	150	10	61	0·58	1·0	3·8	9·7	0·36
22	Pancreatic extract	158	14	40	0·38	0·65	4·4	12·2	0·55
23	Choline	157	12			10·1	3·4	5·0	0·17
24	Choline	156	14			7·4	3·8	7·2	0·26
25	Choline	155	14			5·4	3·6	7·7	0·28
26	Choline	152	13			3·4	4·0	10·1	0·41
				Exp. 4					
27	—	147	13				4·9	17·0	0·91
28	Pancreatic extract	141	12	50	0·48	0·82	4·4	13·3	0·60
29	Pancreatic extract	146	7	25	0·24	0·41	4·3	13·5	0·63
30	Pancreatic extract*	139	9	26	0·32	0·70	5·0	11·0	0·55
31	Choline	144	7			3·5	4·3	9·6	0·41
32	Choline	143	9			2·3	4·5	13·7	0·64
33	Choline	136	10			1·4	4·6	14·6	0·71
34	Choline	146	7			0·7	4·9	15·9	0·78
				Exp. 5					
35	—	158	3				5·3	16·3	0·98
36	Pancreatic extract	166	2		0·24	0·4	4·7	14·4	0·71
37	Choline	161	4			3·5	3·9	13·1	0·53
38	Choline	168	3			2·1	4·5	13·5	0·71

* Filtrate from precipitation with trichloroacetic acid.

Table V. *Exp. 6. Comparative activities of choline and pancreatic extracts on glyceride deposition in the "cholesterol" fatty liver*

Group	Supplement administered	Average initial body wt. g.	Wt. loss % initial body wt.	Extract administered Actual wt. rat day mg.	Pancreas equivalent g.	Extra choline intake rat day mg.	Liver % final body wt.	Total lipoids % fresh liver	In liver of 100 g. rat g.	Glyceride % fresh liver	In liver of 100 g. rat g.
39	—	165	5				8·6	42·3	3·60	36·6	3·11
40	Pancreatic extract	178	7	19	0·40	0·6	6·2	30·06	1·89	24·7	1·53
41	Pancreatic extract	167	7	3·7	0·08	0·1	6·5	35·8	2·38	29·8	1·98
42	Choline	164	5			2·1	5·9	31·6	1·85	25·7	1·51

choline per 100 g. pancreas, while its actual choline content corresponded to 311 mg. Its activity is thus 1·3 or 1·7 times that of its contained choline.

The impossibility of differentiating between the effects of different doses of a supplement unless they differ significantly in amount, which was discussed for groups 12–14 of Exp. 2, is again illustrated in Exp. 3 by groups 19–21 receiving pancreatic extract and groups 23–25 receiving choline. In order not to favour the pancreatic extract choline group 26 is equated with group 20, even though the amount of fat is lower in group 21, which received a smaller pancreas equivalent. Calculating as previously described, the pancreatic extract of Exp. 3 has an action per 100 g. pancreas equal to 442 mg. choline as compared with its content of 171 mg.

The extract used for Exp. 4 was the same as that of Exp. 3. In addition group 30 received the material obtained from the filtrate after precipitating the extract with trichloroacetic acid as described earlier. Both the pancreatic groups 28 and 29 may be equated against the choline group 32. If this be done the extract has an activity equivalent to 479 or 958 mg. choline per 100 g. pancreas. The former of these two amounts is similar to the 442 mg. found for this particular extract in Exp. 3 as described above. These two results, one twice as great as the other, illustrate the limitations of the method when applied to small groups of 10 animals. The trichloroacetic acid filtrate of the pancreatic extract appears to retain all the original activity, for group 30, when equated against group 32, gives an activity equal to that of 712 mg. choline. For the pancreatic extract used in Exps. 3 and 4 the non-choline activity found is thus 442, 479 and 958 mg. choline, and for the trichloroacetic acid filtrate 712 mg.

In Exp. 5, group 36, receiving pancreatic extract, has given a result identical with that of the choline group 38, corresponding to 875 mg. choline as compared with its choline content of 180 mg. During this experiment two other groups of animals received the material of the trichloroacetic acid filtrate obtained by direct extraction of the original pancreas after removal of the trichloroacetic acid as described for Exp. 4. The results which are not recorded in the tables suggested that this method of direct extraction of the pancreas was not as effective as the alcoholic extraction used in the other experiments.

In Exp. 6, pancreatic group 40 may be equated with choline group 42. The activity of this extract is thus 525 mg. choline per 100 g. pancreas; its choline content was 153 mg.

<center>DISCUSSION</center>

Data are presented in Table VI which emphasize the more important points which need discussion. The weight of pancreas equivalent to the volume of extract administered and the actual solids of the ingested extract are recorded as before. Column (c) records in terms of choline the calculated non-choline activity of the pancreatic extract administered, and is obtained by subtraction of the "extra choline intake" of the pancreas groups of Tables II–V from the particular choline dosage with which each extract dosage was equated. Column (d) then expresses the non-choline activity of each extract referred to 100 g. pancreas, expressed in terms of mg. choline.

During the six experiments separate extracts were used in Exps. 1, 2, 5 and 6; a fifth extract was used both for Exps. 3 and 4, and also a trichloroacetic acid filtrate of this extract in Exp. 4. As shown in Table VI, it is possible to assess the activity of the five extracts in terms of choline on eight occasions. After allowing for the actual choline contents of the extracts, which had been previously determined in every case, all the extracts had an action in preventing

Table VI. *Assessment of the activity of pancreatic extracts in terms of choline*

	Extract administered		Choline equivalent of the non-choline activity of the pancreatic extract administered	Non-choline activity of extract/100 g. pancreas
Exp.	Pancreas equivalent/rat/day g. (a)	Solids mg. (b)	mg. (choline) (c)	mg. (choline) (d)
1	0·44	24	3·3	757
2	0·74	93	0·8	108
	0·37	46	0·8	216
3	0·77	80	2·1	273
4	0·48	50	1·5	308
	0·32*	26	2·1	670
5	0·24	—	1·7	704
6	0·40	19	1·5	375

* Filtrate from precipitation with trichloroacetic acid.

fat deposition in the liver varying from an equivalent of 108 mg. in Exp. 2 to 757 mg. in Exp. 1, when expressed as mg. choline per 100 g. pancreas (column (d), Table VI).

The method of assay is not as accurate as could be desired but care has been taken in each case to assess the activity of the pancreatic extracts at a minimum. For example, in Exp. 2, group 7 was equated with group 14, giving a non-choline activity of 108 (Table VI). Groups 12 and 13 however both gave the same amount of fat in the liver as group 14, and had group 7 been equated with either of these, the non-choline activity would have been assessed as 432 and 216 mg. choline per 100 g. pancreas, amounts four times and twice as great. Similarly, if in Exp. 3 group 21 and not group 20 had been equated with choline group 26, the activity found would have been 586 mg. instead of 442 mg. choline per 100 g. pancreas. The average value for the non-choline activity in the eight assays of the 5 extracts corresponds to 426 mg. choline per 100 g. pancreas. It might be objected that since the food intakes were not measured save in Exp. 1, only the results of that experiment are valid, for if the choline groups consumed less food than the 8 g. daily on which the assays are based, clearly the activities of the pancreatic extracts would be assessed at too high a figure. This objection cannot be sustained. In the first place, the food intakes of the choline groups in Exp. 1 are higher than those of the pancreatic groups, and it has been our general experience that these fatty diets are better consumed when choline supplements are added. Secondly the food intakes of the choline groups necessary to reduce the non-choline activity of the extracts to zero, may be calculated. Such calculations show that the amounts would have to be so low as 5·9 and 4·8 g. in Exp. 2, 3·1 g. in Exp. 3, 2·8 and 1·4 g. in Exp. 4, 1·5 g. in Exp. 5 and 2·3 g. in Exp. 6. Since the weight losses of the choline groups were similar to those of the pancreas groups, such values for food intake are clearly impossible. We think it probable that the assumption of a uniform food intake for all the groups has in fact operated against the pancreas rather than·in its favour, as it would have done had it been made in Exp. 1.

It is of interest to calculate the percentage of the total activity of each extract which is due to its choline content. This value ranges from 0 % in Exp. 1 to 74 % in Exp. 2 with a mean value of 35 %. Thus the choline content of the

extracts accounts for only one-third of their activity. Since this is, as already pointed out, a minimum estimate, the presence of a non-choline factor in pancreas appears established, thus extending the claims of Dragstedt *et al.* [1936, 1, 2] to the dietary fatty liver.

While the *non-choline* activity of the extracts, 426 mg., is about $1\frac{1}{2}$ times the actual choline content of the whole pancreas, it is to be admitted that this represents a low degree of activity if the substance is a hormone. The probable explanation is that the method of extraction is inefficient, but in view of the conflicting evidence now to be reviewed, we thought it better to establish the presence of the substance before attempting to improve its extraction.

Since proteins exert a preventive action in liver fat deposition [Channon & Wilkinson, 1935; Beeston *et al.* 1935; Best *et al.* 1936; Channon *et al.* 1938], it is possible that the extra activity of the extracts may be due to their content of protein. In Table I it is shown that the solids of the extracts of Exps. 1 and 2 contained 10·9 and 11·8% total N, and 8·0 and 10·8% non-protein-N respectively. In Exp. 1 the protein-N was, therefore, 2·9% corresponding to 18·1% of protein, and of the 24 mg. actual solids administered 4·3 mg. were protein. If the activity were due to protein it may be calculated that the protein would possess an activity equal to 767 mg. choline per g. Similar calculation shows that in Exp. 2, 1 g. of protein would have the activity of 138 mg. choline. Of many proteins studied [Channon *et al.* 1938] none has an activity significantly greater than that found by Beeston *et al.* [1936] for caseinogen, i.e. 7 mg. choline per g. Clearly, therefore, a protein cannot be responsible for the non-choline effect of the pancreas unless it possesses an activity vastly greater than that of any yet encountered. Further in a single observation in Exp. 4 the trichloroacetic acid filtrate was found to retain all the original activity of the extract. It must not be overlooked, however, that the active substance may have been present in the pancreas originally as part of a protein molecule and liberated by enzymic action during the earlier stages of preparation of the extracts.

The work of Dragstedt and his colleagues has been much criticized, and failure to repeat his results both on depancreatized dogs and on rats has been reported by a number of workers. Chaikoff & Kaplan [1937] have drawn attention to the impossibility of making comparative studies of the effects of choline and pancreatic extracts in any quantitative manner by the histological methods which Dragstedt employed. They have further shown that the fat distribution in the livers of depancreatized dogs varies from lobe to lobe, and therefore that results based on histological examination of biopsy samples are unreliable. Using depancreatized dogs, Kaplan & Chaikoff [1937] were not satisfied that pancreatic extracts had an effect other than that of their choline content. They point out that until properly designed experiments have been carried out in which the choline contents of the extracts are determined and taken into account in any assays, the results of Dragstedt *et al.* [1936, 2] cannot be accepted.

Aylward & Holt [1937] fed groups of rats on a diet causing fatty livers; other groups received the same diet with the addition of raw pancreas or corresponding amounts of choline calculated from the figures for ox pancreas recorded by Fletcher *et al.* [1935]. They concluded that the effect of raw pancreas was adequately explained by its choline content. Study of their results, however shows that the amounts of pancreas and choline fed were such that the "fat" percentage in the livers was in our opinion only sufficiently high at one pancreas dosage for any adequate comparison to be made between the effects of the two

supplements; in addition they based their conclusions on the percentage of fat in the livers only and took no account of the changes in liver size. In this one result, calculation from their data of the amount of fat in the liver of the 100 g. rat shows that 1 g. of pancreas had an action approximately equal to that of 3·5 mg. choline which may be contrasted with the 2·3 mg. choline per g. of ox pancreas reported by Fletcher *et al.* [1935]. Owing to the possible lipotropic effect of the proteins of the pancreas fed, this single result provides but slight evidence as to the existence of an active pancreatic factor other than choline, especially since raw pancreas was fed, and any active non-choline compound in it may have been inactivated by enzyme action. MacKay [1937] administered extracts prepared by Dragstedt's method to rats receiving a diet causing fatty livers in amounts equivalent to 1·2–30 g. of pancreas per rat per day. The livers of the control animals contained 21·5 % fat, and no significant change occurred until the animals received the equivalent of 3 g. of pancreas per day, when it fell to 13·2 %. The author deduced that the pancreatic extract was active in preventing fat deposition, and, holding the view that the extract could not contain enough lecithin or choline to give the experimental results quoted, attributed its action to a non-choline factor. As shown in Table 1, however, such extracts may contain much choline, and indeed in a later paper [MacKay & Barnes, 1938], the same worker attributes half the activity of such extracts to their contained choline. Since the choline contents of the extracts were not determined, while in addition the extracts from very considerable amounts of pancreas were found necessary in order to demonstrate an effect, it is difficult in our opinion to draw any conclusions from these results. MacKay & Barnes [1938] reported further experiments in which extracts equivalent to very much smaller amounts of pancreas (up to 1 g. per rat per day) were found active. Finding an approximately equal distribution of activity in the filtrate and precipitate obtained by the addition of 20 vol. of alcohol to their extract, they regarded this as evidence that both the choline and the protein present in pancreatic extracts act to prevent fat deposition in the liver. In contrast to the previous finding [MacKay, 1937] they concluded that there is no specific pancreatic substance, the action of such extracts being the sum of the activities of the choline and protein. Since the crude alcohol precipitate was regarded as protein, and the choline content of neither fraction was estimated, this deduction appears unwarranted. Further, the liver fat percentages in the control groups of two of their three experiments, 8·2 and 7·0, are in our experience far too low to enable any adequate demonstration of the activity of a supplement to be made.

The only existing claim of confirmation of Dragstedt's results occurs in a preliminary note by Shapiro & Wertheimer [1937]. In this work each rat received extract, of undetermined choline content, equivalent to 14–22 g. pancreas per day, in contrast with the maximum of 0·9 g. used in the present work. From diffusion and adsorption experiments, the authors present some evidence which may be taken as indicating the non-identity of choline itself with the active substance.

For the reasons outlined above, it appears to us that the work which has so far been reviewed neither proves nor disproves the existence of a pancreatic non-choline factor influencing liver fat.

Quite recently Dragstedt *et al.* [1938] have adduced further evidence in confirmation of their original findings.

Best & Ridout [1938] have just reported experiments in which they used a pancreatic extract proved active on depancreatized dogs by Dragstedt; their

results lead them to the conclusion that "lipocaic" has no action on rats beyond that to be expected from its content of choline and protein, and that its activity is no more than that of an equal weight of dietary caseinogen, namely 5 mg. choline per g. From the data presented in this paper, values for the lipotropic value of the solids of our extracts may be calculated: such values range from 33 to 138 mg. choline per g. with an average of 72 mg. per g., which is 14 times that accepted by Best & Ridout [1938] for caseinogen. Since our extracts contained appreciable amounts of choline while the "lipocaic" used by Best & Ridout [1938] contained only traces, it would be fairer to calculate the non-choline lipotropic values: these vary from 9 to 138 mg. per g. and average 54 mg. per g., a figure 10 times that found by the Toronto workers. Their opinion is thus in marked and perplexing contrast to that presented in this paper, and in view of the very considerable body of consistent evidence which we offer, we feel that the explanation of the divergence must await further experiment. The only suggestion we offer is that possibly the single preparation of "lipocaic" administered by Best & Ridout [1938] to each of the two groups of rats used had become inactive subsequent to its being tested by Dragstedt: for this reason it would have been of value had it been possible for the preparation after being found inactive on rats at Toronto, to be re-tested for its activity on depancreatized dogs by Dragstedt.

If the conclusions of Best & Ridout [1938] and of Dragstedt *et al.* [1936. 2] are confirmed they will provide a further example of differences between the factors influencing the dietary fatty liver and those affecting the diabetic fatty liver. One such difference is indicated by the original results of Allan *et al.* [1924] and by numerous later studies: the high protein diets on which depancreatized dogs are maintained fail to prevent the development of fatty livers in these animals, yet it is the protein content of diets low in choline which controls the degree of fat infiltration in the rat [Channon & Wilkinson, 1935; Beeston *et al.* 1935; Best *et al.* 1936; Channon *et al.* 1938]. It would, however, be a curious fact if in diabetic dogs developing fatty livers on diets of high protein content, the addition of raw pancreas controlled such fat infiltration by virtue of the additional protein provided, unless the pancreatic protein is of specific nature: alternatively the pancreas must contain some active non-protein substance other than choline.

SUMMARY

1. Dilute alcoholic extracts of pancreas have been prepared according to the method of Dragstedt *et al.* [1936. 2] and the amount of choline in them determined. The action of each extract in preventing fat deposition in the livers of rats has been assayed in terms of choline.

2. Six experiments were carried out on 42 groups of rats, each group consisting of 10 animals. Three of these experiments were concerned with the "cholesterol" and three with the "fat" fatty liver.

3. Eight assays of the activity of the five pancreatic extracts used were made; all of them showed the extracts to possess an ability to prevent fat deposition in the liver greater than could be attributed to their content of choline. The average value for this non-choline activity was equivalent to that of 426 mg. choline per 100 g. pancreas while the choline present in the extracts accounted for only one-third of their activity.

4. The non-choline activity is not accounted for by the protein content of the extracts and it is concluded that there exists in pancreas a substance other than choline which is involved in fat deposition in the liver.

5. Some of the difficulties of assaying pancreatic extracts are pointed out, and the conflicting evidence on the problem of the activity of such extracts is discussed.

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CLXXVIII. THE EFFECT OF CHOLESTEROL FEEDING ON LIPOID DEPOSITION IN THE LIVER OF RATS

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WHEN rats are fed on diets containing cholesterol, they develop fatty livers which are characterized by the presence in the liver of excessive amounts of cholesteryl esters, while at the same time considerable deposition of glyceride may occur. This variable increase in the glyceride content of the "cholesterol" fatty liver has been shown to depend on a number of factors, such as the dietary contents of choline [Best *et al.* 1934], of caseinogen [Channon & Wilkinson, 1935; Beeston *et al.* 1935], of methionine [Channon *et al.* 1938] and of certain choline analogues [Channon *et al.* 1937, 1, 2]. While the nature of the "cholesterol" fatty liver and the effects on its composition of a number of dietary and other factors have thus been considerably studied, the part played by the fat content of the diet has not been defined. The work described in the present paper has been designed to investigate the quantitative changes which occur in the "cholesterol" fatty liver, as a result of variations in the fat content of the diet, with particular reference to the deposition of cholesterol and of glyceride on diets of constant cholesterol content.

EXPERIMENTAL

The general method of experiment was to feed groups each of 10 rats on diets containing varying amounts of beef dripping with and without 2% cholesterol. In Exp. 1 all the animals were does and the period of feeding was 21 days. The four control groups received a diet consisting of caseinogen (alcohol-extracted) 8, marmite 5, salt mixture 5, cod liver oil 1, with 10, 20, 30 and 40 parts of beef dripping respectively, each diet being made up to 100 parts with glucose. Four other groups received similar diets with the substitution of 2 parts of cholesterol at the expense of glucose. The food intake of the animals was not measured. After 21 days the animals were guillotined and the pooled livers of each group were treated by the alcohol-ether method, and analysed for phosphatide, glyceride and free and combined cholesterol [Best *et al.* 1934]. In Exps. 2 and 3 control groups were omitted. In Exp. 2 the cholesterol-containing diets were identical with those of Exp. 1 save that the caseinogen content of the diet was decreased to 5% and the glucose correspondingly increased. The animals were bucks and the duration of the experiment 17 days. The livers of the animals were investigated individually, and the amount of the fatty acids and unsaponifiable matter determined together by the saponification method. The materials obtained from the livers of the animals of each group were then combined and analysed for their content of unsaponifiable matter, the fatty acids being obtained by difference. In group 12 (Table II) three animals in which the weight losses were greater than 20% were rejected. In Exp. 3 the

diets were the same as for Exp. 2, save that the fat contents of the four diets were 5, 15, 25 and 35 parts respectively, the glucose being correspondingly increased. The animals ($\male\male$) were fed for 21 days, and the pooled livers were worked up by the alcohol-ether method; the total ethereal extract was analysed for fatty acids and unsaponifiable matter only, the sterol content of the latter being determined by the digitonin method. The essential data are recorded in Tables I-III, in which the amounts of the different constituents are recorded both as percentages of the fresh liver weight and as the weight in the liver of the 100 g. rat.

RESULTS

Since the control groups, 1a–4a of Exp. 1, provide data concerning the effect of progressive increases in the fat content of the diet on the nature of the resulting fatty liver, they will be discussed together. Mention is first necessary of findings with normal animals. Work of the last few years has shown that an increasing number of dietary factors are concerned in the control of liver fat. The consequent growing realization of the complexity of liver fat control makes it uncertain as to what should be regarded as "normal" values. For comparison with groups 1a–4a, the liver of the "normal" animal on a balanced mixed diet is regarded as being 3·4 % of the body weight and as containing lecithin 3·3, cholesterol 0·25–0·30, cholesteryl esters 0·03 and glyceride 1·0 % of the fresh liver weight. For the 100 g. rat, these latter figures entail the presence in the liver of lecithin 111, cholesterol 8–10, cholesteryl oleate 1 and glyceride 34 mg. (total fatty acids 112 mg.).

Table I shows that increase in the fat content of the diet causes in general increases in the size of the liver, although these increases do not run strictly parallel with the increasing fat content. Similarly the phosphatide figures show decreases in the percentages of lecithin with increasing fat infiltration, although group 3a contains less lecithin, 2·30 %, than group 4a, 2·63 %. On the basis of the 100 g. rat, however, the livers contain similar amounts of phosphatide 112, 114, 103 and 105 mg. respectively, and these do not differ sufficiently from the normal 111 mg. to be of significance. The percentage of free cholesterol, 0·21, is constant throughout, while the increase in liver size is not sufficiently great significantly to increase its absolute amount. More pronounced changes occur in cholesteryl ester fractions which increase from the normal 0·03 % and 1 mg. to a maximum of 0·33 % and 14 mg. The most striking changes occur, however, in the glyceride fractions which show successive increases from 4·10 to 17·54 % (normal 1 %) as the fat content of the diet is progressively increased. The absolute weight in the liver of the 100 g. rat thus progressively rises to a maximum of 0·722 g., the amounts present in the 11, 21, 31 and 41 % fat groups respectively being about 4, 9, 15 and 21 times greater than the normal. Noteworthy also is the fact that the glyceride fraction whether expressed as a percentage of the liver or in terms of absolute weight, shows a linear increase with the amount of fat in the diet.

The results of the cholesterol groups 1b–4b will now be compared with those of the control groups just discussed. In all cases the liver weight is increased considerably in the cholesterol groups compared with the corresponding control groups. The greatest increase is shown in group 4b in which the liver constitutes 6·88 % of the body weight, a 70 % increase over the control group 4a, 4·12 %. While the inclusion in the diet of 2 % cholesterol thus has a profound effect on the liver size at all levels of dietary fat, among themselves the cholesterol

groups do not increase regularly in this respect, for group $3b$ at $4\cdot97\%$ is lower than group $2b$, $5\cdot24\%$. In the control groups $3a$ and $4a$ a similar inconsistency in this matter of liver size is shown. In every case also the percentages of lecithin are lower than those of the control groups, the extremely low figures of $1\cdot62$ and $1\cdot14\%$ occurring in groups $3b$ and $4b$. While the actual weights of lecithin in groups $1b$ and $2b$, 118 and 115 mg. respectively, are normal, they are significantly decreased in the former groups to 81 and 77 mg. It has usually been found in this laboratory in experiments on the "cholesterol" fatty liver, that the absolute amounts of lecithin are unchanged with progressive infiltration. Thus Beeston et al. [1935] carried out four experiments, in the control groups of which the total weight of lipoids in the livers varied from $1\cdot720$ to $0\cdot618$ g. Reduction of these amounts to $0\cdot281$ and $0\cdot528$ g. respectively by large doses of choline (about 75 mg. per rat per day) had no effect in increasing the absolute weight of lecithin in the liver although the percentages were of course increased. Similarly in other experiments which provided considerable data on this point [Channon et al. 1937, 1, 2] the same result held. The results with groups $3b$ and $4b$ are thus contrary to the general experience in this laboratory.

The effect of the inclusion of cholesterol in the diets is most marked on the cholesterol and glyceride fractions. The free cholesterol shows increases to percentage figures which are 25–50 greater than those of the control groups, while the actual weight in the livers is approximately doubled. These changes are, however, small compared with those in the cholesteryl ester fractions, which increase from the control figures of $0\cdot14$, $0\cdot32$, $0\cdot31$ and $0\cdot33\%$ in groups $1a$–$4a$ to $1\cdot88$, $2\cdot44$, $3\cdot14$ and $3\cdot86\%$ in the corresponding cholesterol groups. The actual weights of cholesteryl ester in the liver of the 100 g. rat thus pass from $0\cdot071$ to $0\cdot265$ g., amounts which are 14, 11, 11 and 18 times greater than those of the corresponding control groups. The amounts of glyceride in groups $1b$–$4b$ also provide clear evidence of the influence of cholesterol in increasing the glyceride fraction of the liver. In all cases, the percentages of glyceride present in the livers of the cholesterol-fed animals are much greater than in the control groups, the minimum increase being in group $1b$ in which it is increased by 10·33 units over group $1a$, and the maximum being in group $2b$ in which it is 20·3 units % greater than in its control group $2a$. It might have been anticipated that the amounts of glyceride in the cholesterol-fed animals would progressively increase as the fat content of the diet increased. This, however, is not the case, for although the percentages and amounts of liver glyceride in groups $2b$ and $3b$ lie between those of groups $1b$ and $4b$, they are not in ascending order. Since in the control groups the amounts of liver glyceride show a linear relationship to the fat content of the diet and in the cholesterol groups the cholesterol contents of the livers do likewise, it seems improbable that group variations are responsible for these irregularities. Accordingly two further experiments were carried out with the object of obtaining further evidence concerning the extent of the deposition of glyceride caused by cholesterol feeding in diets of increasing fat content, and secondly to confirm the progressive increases in cholesterol deposition in the liver observed in Exp. 1. Since both these objects appeared to be obtainable without the use of control groups no such groups were used in Exps. 2 and 3. Before presenting the results of Exps. 2 and 3 in Table II, one point needs mention. In Exp. 2 the amount of cholesterol present in the unsaponifiable fraction of the livers was not determined. Since in cholesterol-fed animals cholesterol constitutes some 90% of the liver unsaponifiable fraction, the extreme limits in Exp. 3 being 88–92%, for the purpose of this discussion the unsaponifiable fraction will be regarded as cholesterol. The amount of free cholesterol in the

Table I. *Exp. 1. The effect of the inclusion of cholesterol on liver lipoids in diets of varying fat content*

Group	Fat in diet %	Cholesterol in diet	Liver (% body wt.)	Liver lipoids (g. per 100 g. fresh liver)					Liver lipoids (g. per liver of 100 g. rat)				
				Lecithin	Cholesterol	Cholesteryl oleate	Glyceride	Total	Lecithin	Cholesterol	Cholesteryl oleate	Glyceride	Total
1a	11	—	3·62	3·11	0·21	0·14	4·10	7·56	0·112	0·007	0·005	0·148	0·272
1b	11	2	4·00	2·95	0·32	1·88	14·43	19·58	0·118	0·014	0·071	0·577	0·780
2a	21	—	3·84	3·05	0·21	0·32	8·25	11·83	0·114	0·008	0·012	0·317	0·451
2b	21	2	5·24	2·19	0·27	2·44	28·55	33·45	0·115	0·014	0·128	1·496	1·753
3a	31	—	4·49	2·30	0·21	0·31	11·22	14·04	0·103	0·010	0·014	0·505	0·632
3b	31	2	4·97	1·62	0·28	3·14	24·14	29·18	0·081	0·014	0·156	1·191	1·442
4a	41	—	4·12	2·63	0·21	0·33	17·54	20·71	0·105	0·009	0·014	0·722	0·850
4b	41	2	6·88	1·14	0·28	3·86	34·40	39·68	0·077	0·019	0·265	2·367	2·728

Table III. *Exp. 3. The effect of the inclusion of cholesterol on liver lipoids in diets of varying fat content*

Group	Fat in diet %	No. of rats	Wt. gain or loss %	Liver % body wt.	Liver lipoids								
					g. per 100 g. fresh liver				g. per liver of 100 g. rat				
					Fatty acids (a)	Unsap. matter (b)	Cholesterol	Total (a − b)	Fatty acids (c)	Unsap. matter (d)	Cholesterol	Total (c + d)
13	6	10	−4·7	4·00	15·94	2·49	2·21	18·43	0·638	0·096	0·085	0·734
14	16	10	−4·7	4·00	17·70	3·01	2·59	20·71	0·709	0·120	0·103	0·829
15	26	10	−8·7	4·37	24·70	3·26	2·83	27·96	1·078	0·140	0·122	1·218
16	36	10	−5·7	4·96	25·37	3·78	3·29	29·15	1·284	0·192	0·167	1·476

"cholesterol" fatty liver being small and subject to relatively little change, changes in total cholesterol represent substantially changes in cholesteryl esters. The results are presented in Tables II and III.

Table II. *Exp. 2. The effect of the inclusion of cholesterol on liver lipoids in diets of varying fat content*

Liver lipoids

Group	Fat in diet %	No. of rats	Wt. gain or loss %	Liver % body wt.	g. per 100 g. fresh liver			g. per liver of 100 g. rat		
					Fatty acid	Unsap. matter	Total	Fatty acid	Unsap. matter	Total
9	11	10	− 7·9	4·20	14·04	1·61	15·65	0·588	0·072	0·660
10	21	10	− 10·4	3·94	14·23	2·75	16·98	0·543	0·106	0·649
11	31	10	9·7	5·88	29·76	2·35	32·11	1·748	0·135	1·883
12	41	7	− 11·1	5·81	29·63	2·85	32·48	1·732	0·156	1·888

The results presented in Table II call for comment on two aspects only, for in general they confirm those of Exp. 1. First, with regard to the cholesterol content of the liver, the percentage in Exp. 2 rises from 1·61 on the diet containing 11% fat to 2·85 on that with 41% fat. The percentages in groups 10 and 11, while lying between those of groups 9 and 12, are not however in ascending order. In Exp. 3 the cholesterol increases regularly from 2·21% in group 13 (6% fat) to 3·29 in group 16 (36% fat). In spite of the irregularity of the percentage figures in groups 10 and 11 in Exp. 2, the actual weights of cholesterol in the livers show again progressive increases in both experiments, from 0·072 to 0·156 g. in Exp. 2 and from 0·085 to 0·167 g. in Exp. 3. These amounts include of course the free cholesterol present, which, as shown in Exp. 1, is about 10 mg. The results of Exps. 2 and 3, therefore, confirm the finding of Exp. 1 that on diets of constant cholesterol but of increasing fat content the amount of cholesterol appearing in the liver is directly related to the fat content of the diet.

Considering now the fatty acid content, Table II shows that in Exp. 2 the four groups fall into pairs, i.e. groups 9 and 10 with 11 and 21% fat in the diet contain 14·04 and 14·23% fatty acids while groups 11 and 12 with 31 and 41% fat in the diet contain 29·76 and 29·63% fatty acid in the liver. The actual weights of fatty acids in the livers behave similarly. In Table III, Exp. 3 shows a similar phenomenon as regards the percentage figures, for groups 13 and 14 have 15·94 and 17·70% fatty acid in the liver, while groups 15 and 16 contain 24·70 and 25·37% fatty acid. The actual weights of fatty acid in this experiment, however, show a progressive increase with increasing dietary fat, although this is not as regular as in the control groups of Exp. 1.

DISCUSSION

The results of the control groups in Exp. 1 show that increasing the fat content of the diet causes linear increases in the percentage and amount of glyceride, a decrease in the percentage but no change in the amount of phosphatide and a small but definite accumulation of cholesteryl esters. The question arises whether the changes in cholesteryl esters are metabolically significant. Previous work has shown that the inclusion of even small amounts of cholesterol in diets results in an increase in the amount of this substance in the liver. Thus Channon & Tristram [1937] found that the daily intake by the 100 g. rat of only 2·82 mg. cholesterol for 21 days caused about a 30% increase in the total cholesterol of the liver, while 9·46 mg. caused a 70% increase. Further, this

deposition of cholesterol as ester, may be detected within a few hours of feeding and is progressive with time, while little change takes place in the free sterol [Aylward et al. 1935]. For these reasons it seemed possible that the increases of cholesteryl ester caused by the increasing fat content of the control diets 1a–4a might be related to the cholesterol content of the beef dripping used in the diets rather than to the changes consequent on the accumulation of glyceride in the liver. While the cholesterol content of the actual fat used in these experiments was not determined, another sample of beef dripping was found to contain 0·2 % cholesterol. Every increase of 10 % in the fat content of the diet would thus result in the cholesterol intake being increased about 1 mg. per 100 g. rat per day, an amount which appears too small to account for the differences observed between group 1a and the other control groups. Work by Minovici [1935] and Eckstein & Treadwell [1935–6] indicates that diets containing unsaturated fatty acids promote sterol synthesis. This finding, if confirmed, may explain the deposition in the liver of small quantities of cholesteryl esters caused by high fat diets, low in lipotropic factors.

With regard to the results of the inclusion of 2 % cholesterol in the diet all three experiments show consistently that the amount of cholesteryl ester appearing in the liver is progressively increased by the fat content of the diet. The simplest explanation of this finding would be that the amount of cholesterol absorbed depends on the amount of fat being absorbed at the same time. Evidence on this question is, however, somewhat scanty and inconclusive. That cholesterol is not significantly absorbed unless fat is present in the diet is shown by the results of Thannhauser [1923] with human beings, Sano [1924] with dogs and Cook [1936] with rats. Some evidence exists also that absorption does not depend on the fat content of the diet. Chanutin & Ludewig [1933] fed two groups of rats on diets both of which contained 2·5 % cholesterol and 4 % cod liver oil, one diet containing 75 % carbohydrate and the other 75 % fat. Surprisingly, the amount of cholesterol in the livers of the animals on the high carbohydrate diet was 6 %, some 4 times greater than that in the livers of the animals receiving 75 % fat in the diet. Cook [1937] found no significant difference in the cholesterol contents of the livers of three groups of rats receiving 2 % cholesterol with 15, 20 and 30 % arachis oil in their diets respectively. The clear contrast between the present results and those of the latter author may perhaps be connected with the chemical nature of the dietary fat used.

One experiment was carried out in which the degree of absorption of cholesterol on a diet containing 40 % fat and 2 % cholesterol was measured. The cholesterol intake was calculated from the daily food intake and the analysis of the faeces of two groups, each consisting of eight animals kept in individual cages, and fed on the same diet with and without 2 % cholesterol. The average cholesterol intake was 132 mg. and the average excretion was 63 mg. per 100 g. rat per day. This shows a daily absorption of about 70 mg. per 100 g. rat which is nearly twice as great as the constant absorption of 0·3–0·4 g. per kg. observed by Cook [1937] in his three groups of animals referred to above. Whilst the accuracy of estimations of the degree of absorption of a substance such as cholesterol by the intake and excretion method is limited, it seems certain that greater absorption occurred in this experiment with 40 % fat, than in that of Cook [1937] with 15, 20 and 30 % arachis oil. It would have been desirable to measure the cholesterol absorption at a series of levels of fat in the diet, but it was not possible to pursue this study further, and until further evidence is available, the progressive increase in the liver cholesterol with increasing fat in the diet must be regarded as the result of increased cholesterol absorption.

It is possible by the addition of supplements of either choline [Best *et al.* 1934] or some of its analogues [Channon *et al.* 1937, 1, 2] or by increasing the protein content of the diet [Beeston *et al.* 1935] to produce "cholesterol" fatty livers containing amounts of glyceride only a few times greater than the normal. It appears reasonable, therefore, to believe that the amount of glyceride present in the liver does not control the deposition of cholesteryl esters. The converse of this does not, however, hold, for the results reported in this paper show that increase in the amount of cholesteryl ester in the liver consequent upon the inclusion of 2 % cholesterol in appropriate diets, is the cause of the marked increase in the amount of the liver glyceride. This increase is apparent at all dietary fat levels, even when the glyceride content of the liver is already high. Thus in the control group, 4*a*, the amount of glyceride, 0·722 g., is some 21 times the normal, yet this is further increased to 70 times normal in group 4*b*, and similar results occur in the other groups.

SUMMARY

1. The effect of varying the amount of dietary fat on the lipoids of the "fat" and "cholesterol" fatty livers has been investigated.

2. On a diet causing "fat" fatty livers in rats, the degree of glyceride infiltration is proportional to the amount of fat in the diet. There is a small increase in the amount of cholesteryl esters which does not appear to be related to the percentage of fat in the diet.

3. On a diet causing the "cholesterol" fatty liver, successive increases in the percentage of fat in the diet cause progressive and large increases in the cholesteryl ester content of the liver. Very considerable increases in the amounts of glyceride also occur, and the "cholesterol" fatty liver always contains much more glyceride than the "fat" fatty liver at any given level of dietary fat.

4. Small increases in the amounts of free cholesterol also occur with increases in the amount of dietary fats, and these become greater when cholesterol is present in the diets.

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CLXXIX. INVESTIGATIONS ON THE NATURE OF HAEMOPOIETIN, THE ANTI-ANAEMIC PRINCIPLE IN HOG'S STOMACH

IV. ON THE BIOCHEMICAL METHOD OF LASCH FOR THE QUANTITATIVE DETERMINATION OF "INTRINSIC FACTOR" IN GASTRIC JUICE

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LASCH [1937] has proposed a method for the early diagnosis of pernicious anaemia based on a test for the enzymic "intrinsic factor" in gastric juice. The extreme importance of such a test for the study of this enzyme and for early diagnosis led us to repeat it under the conditions employed by Lasch and we consider it desirable, at this stage, to record our findings.

The basis of Lasch's test is the hydrolysis of dry preparations of washed protein (beef muscle, liver, fibrin or yeast) by the action at pH 5·5–6 of the enzyme supposed to be present in normal gastric juice. The pepsin is removed by precipitation with caseinogen according to the method of Castle *et al.* [1931]; at the same time, Lasch argues that at pH 5·5, pepsin is in any case inactive. Normal gastric juice and the juices of patients showing achylia gastrica, excluding pernicious anaemia and carcinoma of the stomach, were claimed to have caused hydrolysis of the protein, but in the two last-named diseases proteolysis was said to be either absent or much reduced.

By incubating beef myoglobulin with normal gastric juice and juice from patients with pernicious anaemia and achlorhydric secondary anaemia at pH 6, Griffiths [1934] claimed to have obtained proteolysis with the normal juice only. This has since been criticized by Emerson & Helmer [1936] who showed that the small changes in non-protein nitrogen at pH 6 could have been due to the prolonged action of pepsin surviving irreversible inactivation. Taylor *et al.* [1937] showed that at pH 7·4 under conditions in which the possible actions of pepsin, trypsin and erepsin were excluded, normal gastric juice produces progressive liberation of non-protein nitrogen from caseinogen. They, however, remark that their experiments furnish no proof that this proteolysis is due to the interaction of the intrinsic factor with the caseinogen.

We have carried out the test described by Lasch, using washed dried beef as substrate, on the gastric juice of a small number of patients and, as it soon became evident that no significant difference could be detected by this method between the gastric juices from patients with pernicious anaemia and with other diseases, it was decided that no good purpose could be served by continuing the series. The pH-activity curves for the proteolysis of beef myoglobulin by the juices from normal subjects and cases of pernicious anaemia were also determined.

EXPERIMENTAL

Substrate. Dried beef was made by washing·minced beef twice with dilute acetic acid at pH 5, filtering off the tissue, washing twice with 90 % alcohol, then with absolute alcohol and finally with ether. The beef was dried in an incubator and powdered by passing through a mill.

The test was carried out as follows: a volume of the filtered gastric juice was depepsinized by treatment with sufficient of a solution containing 7·5 % caseinogen and 4 % Na_2CO_3 to bring the pH to 5·5, brought to a convenient volume and filtered.

In each of four stoppered tubes were placed 5 ml. of pH 5·5 acetate buffer solution and 2 drops of chloroform; into the first and second tubes were put 2 ml. of unheated depepsinized gastric juice and 20 mg. beef powder; into the third tube 2 ml. of depepsinized juice and into the fourth 2 ml. of heated (at 100° for 10 min.) depepsinized juice and 20 mg. beef powder. The contents of the first tube were deproteinized at once by the addition of 7 ml. of 15 % trichloro-acetic acid and those of the other tubes after incubation for 24 hr. Next day, the protein precipitate was filtered off through a small Whatman No. 42 paper and the non-protein nitrogen in a portion of the filtrate determined by the micro-Kjeldahl method. It was usually found that the amounts of non-protein nitrogen in the first, third and fourth tubes were equal, showing that no autolysis occurred in the gastric juice when incubated alone and that the heating was sufficient to inactivate any enzyme present. The amount of proteolysis is then given by the increase in non-protein nitrogen in the second tube when compared with the other three.

The results, calculated as mg. nitrogen produced by 1 ml. of juice, obtained in thirteen cases of pernicious anaemia are given in Table I and those from

Table 1. *Cases of pernicious anaemia (achlorhydria before and after histamine stimulation)*

Case	mg. non-protein nitrogen produced per ml. juice	
	Before stimulus	After stimulus
1	0·82	—
2	1·21	4·82
3	1·24	—
4	4·18	1·32
5	0·50	—
6	2·44	2·84
7	0·56	0·02
8	0·00	1·62
9	0·62	1·66
10	0·90	—
11	4·52	3·32
12	Combined juice 0·16	
13	Combined juice 0·36	
Mean	1·65 ⊥ 0·32	

eighteen cases of other diseases in Table II. In most of the cases the test was done on gastric juice obtained before and after stimulation by histamine injection or by gruel. The means with their standard errors for the three groups pernicious anaemia, other anaemias and cases other than anaemia are $1·65 \pm 0·32$, $1·16 \pm 0·17$ and $1·36 \pm 0·13$ respectively and do not differ significantly. The ranges covered by the results from the three groups are 0·00–4·82, 0·00–2·48

Table II. *Cases other than pernicious anaemia*

		Free acid (units)		Hydrolysis: mg. N produced per ml. juice	
		In fasting contents	After stimulus gruel G or histamine H	Fasting contents	After stimulus
Case	Diagnosis				
	Anaemias other than pernicious anaemia				
14	Anaemia of pregnancy	0	16 G	1·31	0·40
18	Hypochromic microcytic	0	0 G	2·48	1·14
21	,, ,,	0	0 G	—	1·30
22	,, ,,	3	33 G	0·68	1·78
23	,, ,,	0	0 G	—	0·00
24	,, ,,	0	0 H	0·52	0·78
25	Aplastic anaemia	11	26 H	2·22	1·46
31	Secondary to cirrhosis	24	45 G	0·54	—
17	Leukaemia with anaemia	0	0 H	—	0·88
20	,, ,,	25	80 H	2·04	1·07
Mean				1·16 ± 0·17	
	Non-anaemic patients				
15	Hyperpiesis	0	33 G	—	1·75
16	Rosacea, flatulent dyspepsia	26	40 G	1·40	1·30
19	Polycythaemia	50	70 H	0·76	1·47
26	Gastric ulcer	21	51 H	1·00	0·66
27	Cirrhosis	0	33 H	1·46	—
28	Thyrotoxicosis	0	0 H	1·92	—
29	Duodenal ulcer	35	38 H	1·25	—
30	Ileitis	35	31 H	2·00	—
Mean				1·36 ± 0·13	

and 0·66–2·00 respectively. The mean value obtained by Lasch for his series of cases of disease other than anaemia was 1·03 ± 0·07 with a range of 0·45–2·7. This differs little from the mean for our group of similar cases. Of his cases of pernicious anaemia, however, twelve out of seventeen gave zero values and five ranged from 0·08 to 0·18. The mean value for his cases of anaemia other than pernicious anaemia was 1·23 ± 0·21 with a range of 0·81–2·73, which also agrees well with our results. We can only conclude that Lasch's test as a diagnostic procedure is unreliable in our hands. The high results obtained for certain of our cases of pernicious anaemia, compared with those for the non-anaemic patients, have to be explained, since it is generally considered that the enzyme content of the gastric juice is diminished in the former. It must be remembered, however, that the juice is also diminished markedly in quantity [Wilkinson, 1932], so that what little peptic or other enzymic activity is present might be more concentrated.

The pH activity curves of gastric juices from normal subjects and cases of pernicious anaemia

The pH activity curves of gastric juice using standard buffered solutions of beef myoglobulin were determined by the method of Jones *et al.* [1938]. The buffered solutions covered a range of pH from 1·45 to 10·8. The results are shown in Fig. 1, in which the circles refer to the juice obtained from a case of pernicious anaemia (Case 13 in Table I), identical figures being obtained for the juice withdrawn before and after histamine stimulation. The triangles refer to combined samples of normal gastric juice. The substrate concentration was 0·56 mg. protein-nitrogen per ml. The ordinates are mg. non-protein-nitrogen produced by 1 ml. gastric juice in 1 hr. and are arranged so that the activity at pH 1·5 is the same for both kinds of gastric juice. It is evident that the

curve is the same for both types of juice and it is very similar to the curves obtained by Jones *et al.* [1938] for the action of pepsin and fractions of hog's stomach on beef myoglobulin and considered tentatively by them as solely due to the pepsin contained in the former two. Determinations were carried out at seven *p*H values between *p*H 5·8 and 10·8, but the activity at these reactions
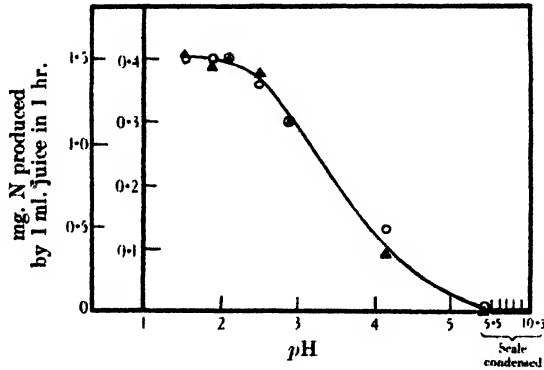
Fig. 1. Activity of gastric juice at different reactions: (1) activity of normal gastric juice (triangles); (2) activity of juice of pernicious anaemia (circles).

of the juice from cases of pernicious anaemia and from normal subjects was zero within experimental error (0·01 mg. N per ml.). The proteolysis obtained by us and other workers at *p*H 6 and 7·4 is probably the result of prolonged digestion, in spite of the great diminution in activity at these reactions.

SUMMARY

1. The method suggested by Lasch [1937] for the determination of the intrinsic factor in gastric juice has been found to be unreliable.

2. The activities at reactions of *p*H 1·5–10·8 of the gastric juices from normal subjects and from patients with pernicious anaemia have been compared and found to follow an identical curve.

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CLXXX. FURTHER OBSERVATIONS ON THE SYSTEM ASCORBIC ACID-GLUTATHIONE-ASCORBIC ACID-OXIDASE

By ERIC MITCHELL CROOK AND FREDERICK GOWLAND HOPKINS

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IN a paper by Hopkins & Morgan [1936] it was shown that in aqueous solutions containing Szent Györgyi's oxidase together with ascorbic acid and glutathione, the former is protected by the latter from oxidation by the enzyme, while the glutathione is itself oxidized vicariously at a rate corresponding to that at which the ascorbic acid is oxidized when alone with the enzyme in similar concentrations. It was also shown that under the influence of similar enzyme preparations the reduction of dehydroascorbic acid by GSH is much faster than the oxidation of ascorbic acid, and also much faster than the reduction when uncatalysed.

Kertesz has since stated [1938] that he was unable to reproduce these experimental results. He found that GSH added to solutions of ascorbic acid containing the oxidase had no effect on the oxidation of the ascorbic acid at pH 6. At pH 7·4, while the GSH inhibited to some extent the oxidation of ascorbic acid, both substances were oxidized simultaneously. He found further that the reduction of dehydroascorbic acid by GSH was not catalysed by the enzyme preparation used by him.

It became clearly desirable that the experiments should be repeated, and the present paper deals with such repetitions together with some extensions. They have involved the use of many different enzyme preparations, and they show that the results published by Hopkins and Morgan are invariably reproducible. The enzyme preparations used by these authors were all derived from cabbages or from cauliflower florets. Kertesz employed cauliflowers, but also used cucumber juice. With no preparation did he obtain results comparable with those of the earlier workers. In the following sections experiments with cauliflower florets will be first described; certain others obtained with cucumber juice will afterwards receive special reference.

Unless an explanation which we will later venture to put forward is justified, we seem to be faced with an inexplicable difference in experimental experience.

EXPERIMENTAL

The cauliflowers employed have been both English-grown and imported. The concentration (or activity) of the oxidase has varied widely in different cases. In general the fresher the source of the supply the greater was the activity of the juice, but it varied apparently with the degree of maturity of the florets. The juice was expressed with avoidance of contact with Fe or Cu, and glass-distilled water was always used for making up solutions. The juice in each case was centrifuged before use, but was not otherwise fractionated. Owing to the wide variations in enzyme concentration we found it desirable to make a preliminary estimation of the rate at which each preparation oxidized a known

amount of ascorbic acid before proceeding to other experiments. The GSH was prepared in the laboratory; crystalline fractions almost free from any tendency to autoxidation being always employed. The ascorbic acid was usually that supplied by the British Drug Houses Ltd., but we have also used a standardized natural product kindly supplied by Sir Henry Dale. Both reactants were more stable than were apparently those employed by Kertesz. The methods of estimation were exactly those used by Hopkins & Morgan and also by Kertesz. A minor difference in procedure was that while our solutions were shaken continuously on a machine, in Kertesz's experiments they were shaken every 2 min. by hand. Our supply of GSSG was made by enzymic oxidation arrested before irreversible products were formed.

Experiments with cauliflower juice

In the work of Hopkins and Morgan, the behaviour of the system—ascorbic acid-GSH-oxidase—was chiefly studied at pH 7·4. It seemed desirable to determine through what range of variation in pH the protection of ascorbic acid and the other results obtained by them (if confirmed) would remain constant. Experiments were therefore made at pH 4·5, 6·0, 6·75, 7·4 and 8·25 respectively. The results are shown in Figs. 1–5.

Fig. 1 shows the relations displayed during aeration at pH 7·4. The enzyme preparation used (from a home-grown cauliflower) was initially very active, 50 mg. ascorbic acid (or, in the system an equivalent amount of GSH) being oxidized in <20 min. It was found that the oxidation of the ascorbic acid began just when a nitroprusside test first showed the disappearance of GSH. In order however that the relations could be more clearly shown (Fig. 1), the juice was diluted before use with twice its volume of glass-distilled water to reduce the velocity of change. The results reproduced exactly those of the earlier studies.
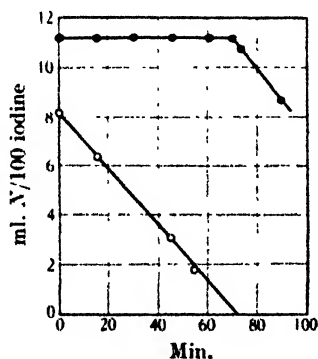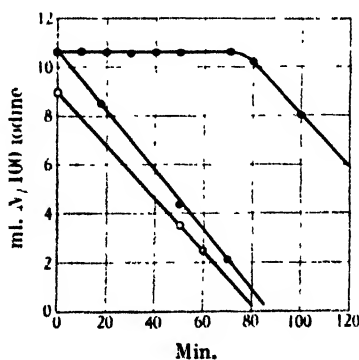
Fig. 1.   Fig. 2.

Fig. 1. Behaviour of system at pH 7·4. Upper line shows protection and subsequent oxidation of ascorbic acid. Lower line, course of oxidation of GSH in system. 1·75 mol. of former to 1 mol. of latter. McIlvaine's buffer (phosphate-citrate).

Fig. 2. System at pH 8·25. 2 mol. GSH to 1 mol. AA. Upper line, protection and subsequent oxidation of ascorbic acid. Middle line, oxidation of GSH in system. Lowest line, oxidation of ascorbic acid with enzyme in absence of GSH. McIlvaine's buffer.

The results at pH 8·25 are shown in Fig. 2. They were given by an undiluted preparation and are essentially the same as those obtained by Hopkins & Morgan, and by ourselves, at pH 7·4.

Fig. 3 shows the behaviour of the system at pH 6·75 and Fig. 4 that at pH 6. Both are again identical in essentials with the above.

Very different is the behaviour at pH 4·5 (Fig. 5). At this more acid reaction the system is dislocated; the protection of the ascorbic acid is imperfect and (as in Kertesz's experiments) both it and GSH are oxidized together.
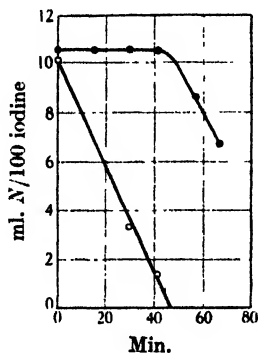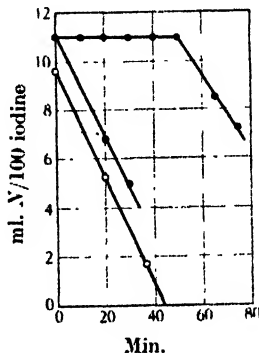
Fig. 3.   Fig. 4.

Fig. 3. System at pH 6·75. Details as in Fig. 1. GSH/ascorbic = 2 mol., 1 mol.

Fig. 4. System at pH 6. Details as in Fig. 3.

It is easy to show from pH-velocity curves that this is because somewhere near pH 5 there is a critical point at which the velocity of the reducing process falls below that of the oxidative one.
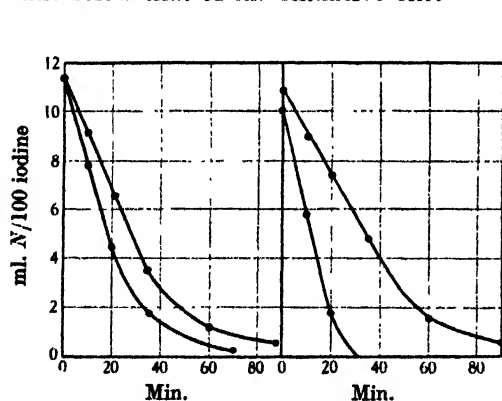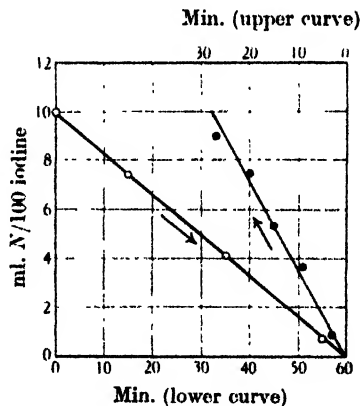
Fig. 5.   Fig. 6.

Fig. 5. System at pH 4·5. Two different enzyme preparations. Upper curve in each case, oxidation of GSH. Lower curves that of ascorbic acid. Ratio GSH/ascorbic = 2 mol./1 mol. McIlvaine's buffer.

Fig. 6. Lower line, enzymic oxidation of ascorbic acid; upper line, catalysed reduction of dehydro-ascorbic acid by GSH. For details see text. Sørensen's or McIlvaine's buffer.

Before the behaviour of the system at these low pH values can be understood, the behaviour of the mechanism which catalyses the reduction of ascorbic acid by GSH must receive reference. Its existence was clearly shown in the experiments described by Hopkins & Morgan, but Kertesz states that he was unable to repeat them. Pfankuch [1934] found that in potato juice the reduction of

dehydroascorbic acid by cysteine required enzymic catalysis for its occurrence as it did not occur in the previously heated juice. The influence of such a catalyst was first clearly shown in the experiments just mentioned.

Fig. 6 shows the relative velocities of the oxidation of ascorbic acid and its reduction by GSH as induced by a preparation of cauliflower juice. 88 mg. natural ascorbic acid in 100 ml. Sørensen's phosphate buffer at pH 6, together with 10 ml. enzyme preparation, were shaken in an open flask. The course of oxidation was followed by titrating successive 10 ml. samples. Oxidation was stopped at a point fixed by extending the strictly linear course to the base line when it could be assumed that reversible oxidation was complete. Samples of 10 ml. each were then transferred to a series of vacuum tubes in the bent-over hollow stoppers of which were 30·7 mg. GSH in 2 ml. buffer. After evacuation the GSH was mixed with the solutions and the tubes allowed to stand at room temperature (21°). The contents of the individual tubes were then titrated at successive time intervals. Oxidation and reduction thus progressed under the enzyme as originally added, but the volume of solution during reduction was greater than during oxidation in the proportion of 12 : 10. It should be noted that in this experiment only 2 mol. GSH were added for each mol. ascorbic acid; yet the reduction rate was linear until the process was almost complete, just as in the experiments which demonstrate that protection lasts till the GSH is practically all oxidized.

The uncatalysed reduction of ascorbic acid by GSH is a phenomenon long familiar in this laboratory. That it is much slower than the catalysed reaction was shown by an experiment—one of many with similar bearings—of which the results are given in Fig. 7. In this case the GSSG was made by oxidizing GSH with iodine. The I_2 was quantitatively removed by lead acetate and a minute excess of Pb by K_2SO_4. Reduction was carried out in vacuum tubes: 4 tubes in each of 3 series. One series contained the enzyme solution, in a second this was replaced by an equal volume of water, in the third each tube contained 0·022 mg. Cu. Otherwise the content of every tube was the same, 3 mol. GSH being present for each mol. ascorbic acid. The pH was 6. The evacuated tubes stood at room temperature (22°) and their contents were titrated at successive intervals. In Fig. 7 the upper curve shows the rate of the enzyme-catalysed reduction, the lower curves that of reduction in the absence of enzyme. Of the latter two, which are nearly identical, the lower shows that Cu does not catalyse the reduction process. With enzyme the dehydroascorbic acid was completely reduced in 15 min. or less, without it only 40 % was reduced in 1 hr. It is the experience of this laboratory that similar results are always to be obtained with the two varieties of *Brassica* that have so far been employed. In his paper Kertesz [1938] remarks "The results obtained with the system shown in their Fig. 3 (i.e. complete protection of the ascorbic acid so long as GSH is present) were observed at pH 7·4. It is doubtful whether the experiment could have been repeated at pH 6, although Hopkins & Morgan make no statement to this effect." Fig. 6 of the present paper shows however that there is complete protection at that pH while the catalysis of reduction at pH 6 is fully confirmed.

It is important to note the general effect of pH on the relative activities of the oxidase and the reducing enzyme (or catalytic system).

Fig. 8 is based on determinations of the initial velocities of oxidation and reduction respectively at varying pH values. They were made with an active specimen of cauliflower juice. The flatter and unsymmetrical curve (A) relates to oxidation velocities, the symmetrical (B) to those of reduction. The points on the curves are from estimations of the amount of change in either direction

induced in 15 min. It is seen that through a range of high pH values reduction is a faster process than oxidation while through a range of lower values reduction is the slower. In the complete system protection of ascorbic acid during the presence of GSH would be expected throughout the former range and not within the latter. In a number of less complete studies we found that the transition point where the curves cross lay between pH 4·5 and 5. The exp. of Fig. 8 was
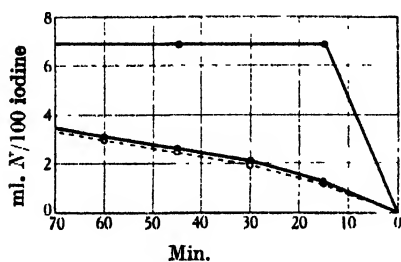
Fig. 7.

Fig. 8.

Fig. 7. Curves run from right to left. Upper, the catalysed reduction by GSH. Lower continuous line, the rate of reduction when uncatalysed. Lower broken line, rate of reduction in presence of copper. Sørensen's buffer.
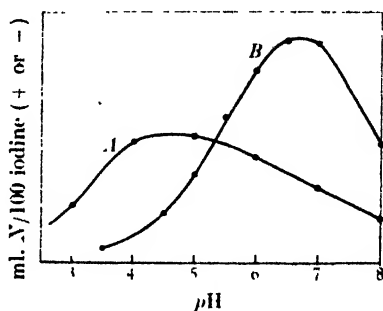
Fig. 8. pH-velocity curves. Curve A gives the initial rates of oxidation measured at different pH values. Curve B applies similarly to rates of reduction in the presence of the enzyme of (enzymically prepared) dehydroascorbic acid. McIlvaine's buffer.

carried out for the construction of more complete curves for publication, and it happened that the transition point in this case was just above pH 5. It would indeed be satisfactory if all the aspects of behaviour displayed by the system could be studied with the same preparation and without delay. This we have found to be impossible. While it is true, as various authors have stated, that the oxidase itself is relatively stable, the reducing mechanism is much less so. Its activity may be lessened in preparations kept overnight, and falls off slowly when an expressed juice is shaken in the air in the absence of any added substrate.

From these considerations it will be understood that in observations made at a pH only a little above 5, the degree of protection may be uncertain. At pH 5·25 for instance we have obtained typical protection with a fresh juice, but none with the same juice after standing for 3 days, though even then there was sufficient of the reducing factors left for protection at pH 7·4.

Fig. 8 makes clear the general nature of the influence of pH variations on the relative rates of oxidation and reduction, and explains completely the disappearance of protection at pH 4·5 (Fig. 5).

Hopkins & Morgan [1936] reported a circumstance involving some difficulty in explanation. Though typical protection was obtained in the system when dialysed juice was employed, dialysis nevertheless diminished the power of preparations to promote reduction. Kertesz [1938] in commenting on this remarks, "The observation that dialysed cauliflower juice could produce complete protection of ascorbic acid in the presence of glutathione but could not catalyse the reduction of dehydroascorbic acid by glutathione makes the explanation given for the mechanism somewhat uncertain." This does not state the facts correctly. With dialysed juice the initial velocity of reduction was as

great as with undialysed, but the rate fell off before reduction was complete. This circumstance still lacks explanation, but the additional evidence given in this paper for the relative instability of the reducing factor (or factors) perhaps affords some help. It is possible that dialysis reduces the amount of some protective agent. As stated above, the reducing activity of a preparation falls off when it is shaken aerobically by itself. This destruction may proceed faster in the dialysed juice.

We venture to include here parenthetically the results of an experiment carried out by Hopkins & Morgan but not published, which shows how justly ascorbic acid might be looked upon as a coenzyme for the oxidation of GSH. Dialysed juice was employed so that all pre-existing ascorbic acid was removed and GSH therefore quite unaffected by the oxidase. In Fig. 9 is shown the effect of subsequently adding minute amounts of ascorbic acid on the rate of GSH oxidation.
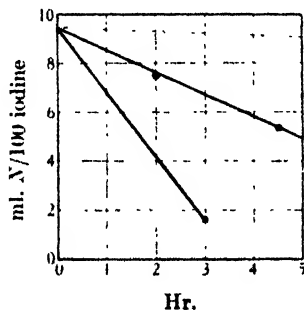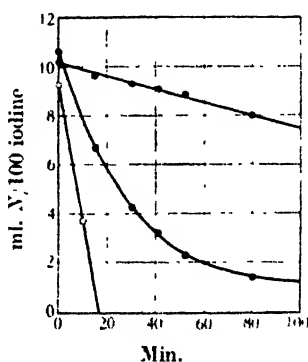
Fig. 9.   Fig. 10.

Fig. 9. Exp. with dialysed juice. Broken line, GSH with enzyme alone (unaffected). Middle line, oxidation of same in presence of 0·1 mg. ascorbic acid. Lower line with 1·0 mg. Sørensen's buffer, pH 7·4.

Fig. 10. System at pH 7·0 with cucumber juice. Upper line, oxidation of ascorbic acid in system. Middle curve, oxidation of GSH in system. Left-hand line, oxidation of ascorbic acid when alone with enzyme. 2 mol. GSH to 1 mol. ascorbic acid. McIlvaine's buffer.

Experiments with cucumber juice

Until the appearance of Kertesz's paper we had had no experience of the use of preparations from the cucumber, which were used in the greater number of his experiments. It is well known that enzyme systems differ widely in plants of different families, and they may well differ in different tissues of the same plant. One thing is sure, however. Unlike some other fruits, e.g. the apple [Johnson & Zilva, 1937], the cucumber contains the ascorbic acid oxidase [Kertesz, 1938]. It also contains the reducing system, but in all the specimens we have employed the concentration of this relative to that of the oxidase is small when compared with their mutual relations in the leaves and florets of the *Brassica* plants. In experiments such as those on which Fig. 8 was based, we have found that the velocity of reduction never rises nearly so far above that of oxidation as is shown in this figure, and it remains above it during a much smaller range of pH values. The maximum velocity of reduction is at a somewhat less acid reaction (about pH 7) than that of oxidation. It further seems sure that for some reason the reducing system is less stable or less protected in

cucumber juice than in that of cauliflower or cabbage. This indeed seems to be the case with the oxidase also. When the two juices without any addition are thoroughly aerated side by side by shaking at pH 6, the resultant falling off of oxidase activity is much more marked in the cucumber juice than in the other. It may be however that a lower relative concentration of the reducing system in cucumber constitutes the main difference between them.

The results we have obtained with cucumbers do not differ fundamentally from those published by Kertesz, except that at pH values near to 7 we have found greater protection by GSH than he reported. At pH 6 it may be small or absent. Fig. 10 gives results typical of those we have obtained at pH 7·0. The departure of the GSH oxidation from the linear progress which was always found in the experiments with cauliflower juice may have been due here to a gradual destruction of the less stable reducing system.

<div align="center">DISCUSSION</div>

In this paper it is shown that all the results obtained by Hopkins & Morgan in their study of the system under reference are reproducible, and we may add that when the experimental conditions are properly defined the reproduction has been in our experience invariable. In so far as he used the same materials as those used by them, the inability of Kertesz to obtain the same results is exceedingly difficult to explain.

The greater number of Kertesz's experiments were however carried out with preparations from cucumbers, and with this material our own results, though not identical with his, come closer to them. It is so well known that the enzymes in plants of different orders or families vary in nature and organization, and so probable that those in different tissues even of the same plant may differ, that it was not justifiable for this author to apply results obtained from material different from that used by Hopkins & Morgan in criticism of their findings. This he does, at least by implication.

It is true that he also used cauliflowers, but apparently in a few experiments only. There remains nevertheless the difficulty of reconciling his results with those of Hopkins & Morgan and our own. It is perhaps possible though very improbable that the explanation might be found in the different provenance of the plants employed; Kertesz's experiments being done in Stockholm. This becomes the less likely since in the Cambridge experiments plants imported from the Continent and others grown on different soils in England have been employed. A more likely explanation is to be found in the instability of the reducing mechanism, of which Kertesz was not aware. He has kindly informed us that the plants used had probably travelled a long distance before they were purchased, and that the expressed juices were not always used immediately after their preparation.

We are particularly concerned to emphasize the quite certain presence in the plants which, because of the nature of Szent Györgyi's pioneer studies, were used by ourselves, of an agent which actively catalyses the reduction of ascorbic acid by glutathione. Its distribution has yet to be explored, but even if this proves to be limited, it is yet an enzyme (or enzyme in association) of which the kinetics must possess a special interest. In any study of these its relative instability must be remembered. An endeavour is being made in this laboratory to separate it from the associated oxidase.

SUMMARY

The experiments of Hopkins & Morgan [1936] on the system ascorbic acid-glutathione-ascorbic acid-oxidase, which Kertesz [1938] was apparently unable to confirm, have been repeated and extended, and the original results completely confirmed. In particular the effect of variations in pH on the enzymic activities of oxidase preparations has been determined.

The expressed juice from cucumbers differs in certain noteworthy respects from that obtained from cabbages and cauliflowers. It contains the oxidase, as Kertesz has shown, and it also contains a mechanism capable of catalysing the reduction of ascorbic acid by glutathione. The latter however is present in relatively low concentration.

Possible reasons for Kertesz's inability to repeat the earlier experiments are discussed.

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CLXXXI. ALCOHOL DEHYDROGENASE OF ANIMAL TISSUES

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IT was first found by Batelli & Stern [1910] that extracts from various animal tissues are able to oxidize alcohol. More recent investigations and attempts to isolate the enzyme responsible for propyl alcohol oxidation were carried out by Reichel & Köhle [1935]. Alcohol oxidation in tissue slices from normal and fasting animals was studied by Leloir & Munoz [1938]. Bernheim [1938] investigated the influence of alloxan on the alcohol oxidation in tissue extracts.

The present work was undertaken in connexion with the study of the liver aldehyde mutase [Dixon & Lutwak-Mann, 1937]. This enzyme catalyses the dismutation of $CH_3.CHO$ to C_2H_5OH and $CH_3.COOH$ and it was of interest to investigate the alcohol dehydrogenase in the liver by methods similar to those employed for the mutase study.

Materials

Horse liver was used throughout as the source of the animal alcohol dehydrogenase. Livers from other animals (dog, pig, sheep) were also found to contain the enzyme in large quantities. A number of preparations were made in which acetone and ammonium sulphate were used for precipitation.

1. *Acetone preparation.* Minced horse liver is mixed with an equal volume of water and after standing a few hours at room temperature the large particles are removed by centrifuging. The aqueous extract is heated to 52° for 15 min. to remove inactive protein, then cooled and precipitated with 3 vol. acetone. The precipitate is centrifuged and resuspended by grinding with a small amount of water. The insoluble part is discarded and the extract is then precipitated with 2 vol. acetone. (Sometimes a 3 : 1 mixture of acetone and alcohol is used.) This procedure is repeated twice. The final acetone precipitate is dried *in vacuo* over $CaCl_2$. This preparation usually contained in addition to the alcohol dehydrogenase an active aldehyde mutase, but gave negative results for the aldehyde oxidase.

2. *Ammonium sulphate preparations.* (a) The aqueous liver extract is precipitated with 3 vol. saturated ammonium sulphate, the precipitate is filtered off and partially dried in air, then completely dried *in vacuo* over H_2SO_4 and KOH. (b) The liver extract is heated to 52° for a few minutes, the resulting precipitate discarded and the filtrate precipitated using 30 g. solid ammonium sulphate for every 100 ml. fluid. (c) The preparation obtained in (a) is taken up in a small quantity of water and dialysed against running tap water for 24 hr. The precipitate formed on dialysis is filtered off and the fluid precipitated with acetone in the same way as described in the acetone preparation.

A number of experiments were also carried out using acetic bacteria as the source of a system capable of alcohol oxidation. Cultures of *Acetobacter suboxydans* grown on glycerol-yeast water medium were spun off, washed twice with water and finally suspended in water. This suspension was used for experiments and was found to retain its activity for several days at 0°.

Coenzymes. Cozymase (70% pure) was prepared from baker's yeast by a modification and combination of Myrbäck's methods as described by Green & Brosteaux [1936]. Coenzyme II from red blood cells and flavoprotein from Lebedew juice were prepared by the methods of Warburg & Christian [1931; 1932]. Cocarboxylase and adenosinetriphosphate were obtained as described by Lohmann [1931] and Lohmann & Schuster [1937].

Methods

The enzymic oxidation of alcohol was tested mainly by two methods. (1) Anaerobically, in presence of methylene blue as hydrogen acceptor following the usual Thunberg technique. Tubes with hollow stoppers were used from which the substrate, methylene blue and other reactants were emptied into the tube containing the enzyme solution after the latter had been exhausted and filled twice with O_2-free N_2, and the reduction time of methylene blue at 37° was measured. (2) Aerobically, O_2 uptake was measured using Barcroft differential manometers with side bulbs [Keilin & Hartree, 1935]. The enzyme solution in $M/30$ phosphate buffer was placed in the right-hand flask only and the phosphate buffer in the other flask. The inner tubes contained KOH-soaked filter papers. Alcohol and other substances were emptied into the flasks from both side bulbs after 15 min. equilibration at 37°.

Properties of the alcohol dehydrogenase system

Coenzymes. The yeast alcohol dehydrogenase has been shown to require for its activity the presence of cozymase (diphosphopyridine nucleotide, coenzyme I) and of flavoprotein (yellow enzyme). In order to determine whether these two substances play a similar role in the oxidation of alcohol by preparations from animal tissues it was essential to have a coenzyme-free enzyme preparation.

Ordinary horse liver acetone preparations were unsuitable for this purpose since they always contain large amounts of cozymase which cannot be removed even by prolonged dialysis [Reichel & Köhle, 1935; Dixon & Lutwak-Mann, 1937]. The last-named authors used dog liver acetone preparations which contain practically no cozymase owing to its quick decomposition by this particular liver tissue. A very convenient and simple method has now been found to obtain horse liver preparations completely free from cozymase. If the liver extract be first treated with ammonium sulphate (as under (*a*), p. 1364) and either used directly or precipitated with acetone after removal of ammonium sulphate (as under (*c*), p. 1364) it then appears to be cozymase-free. On testing these preparations for aldehyde mutase, unless cozymase was added they showed no trace of activity. It can also be seen from Table I, Exps. 1–6, that the alcohol dehydrogenase prepared in this way catalyses the reaction only on addition of cozymase. Coenzyme II (triphosphopyridine nucleotide), cocarboxylase, adenosinetriphosphate, glutathione, known to act as coenzymes in various enzymic

Table I. *Effect of cozymase and flavoprotein on various liver alcohol dehydrogenase preparations*

Thunberg tubes with 20 mg. enzyme preparation, 1 mg. methylene blue, 2 ml. phosphate buffer $M/30$, pH = 7·6.

Ammonium sulphate preparation	ml. alcohol 96%	mg. cozymase	mg. flavoprotein	Reduction time min.
Exp. 1	0·1	—	—	∞
2	0·1	0·02	—	(Several hr.)
3	0·1	0·10	—	35
4	0·1	0·40	—	7
5	0·1	1·00	—	6
6	0·1	2·00	—	6
7	0·1	0·50	2·0	7
8	—	0·50	2·0	∞
Acetone preparation				
9	0·1	—	—	60
10	0·1	0·30	—	20
11	0·1	0·60	—	20
12	0·1	0·60	2·0	18

systems, and also nicotinamide, were found to be completely inactive. Recently Quibell [1938] has also found cozymase to be the specific coenzyme of the animal alcohol dehydrogenase.

That cozymase is also involved in the oxidation of alcohol by the acetic bacteria was shown in the following way. Fresh untreated suspensions of *Acetobacter suboxydans* very vigorously oxidize alcohol, both aerobically and anaerobically, and it is difficult to produce an acceleration of the reaction by addition of cozymase. When, however, the suspension was saturated with ammonium sulphate and after a few hours exhaustively dialysed, a marked increase in the velocity of the reaction was found on addition of cozymase.

Table II. *Effect of cozymase on oxygen uptake in presence of alcohol by acetic bacteria*

In Barcroft manometers, bacterial suspension in phosphate buffer $M/30$, $pH = 6.5$, 2 mg. alcohol, 0.3 ml. 20% KOH in inner tube, total volume 3.3 ml.

	O_2 uptake $\mu l./20$ min.
Fresh suspension	320
Do. +0.4 mg. cozymase	350
Treated with ammonium sulphate and dialysed	90
Do. +0.4 mg. cozymase	200

With regard to flavoprotein it can be seen from Table I, Exps. 7, 12, that it has only very little effect on the reaction. Dewan & Green [1938] were also unable to see any marked influence of flavoprotein in animal enzyme systems.

Coenzyme-factor. Stability of the alcohol dehydrogenase

The belief that the alcohol dehydrogenase from animal tissues is a very labile and unstable enzyme [Batelli & Stern, 1910; Reichel & Köhle, 1935] is disproved by the following observations. Two types of liver alcohol dehydrogenase preparations, (1) the usual acetone preparation, and (2) an ammonium sulphate preparation, made by method (*a*) above, were tested by the methylene blue technique every 2 weeks for a period of 4 months. The crude ammonium sulphate preparation seemed to be the more active one and its activity remained practically unchanged during the long period of observation. The acetone preparation gave lower initial values and seemed to deteriorat egradually on standing. This suggested the possibility of a special agent necessary for alcohol dehydrogenase activity, present apparently in the cruder ammonium sulphate preparation but partly destroyed in the acetone-treated preparation, in which the agent also seemed to undergo progressive decomposition on storing.

At this stage a note was published by Dewan & Green [1937] on the necessity of a special oxidation catalyst which they called the "coenzyme factor" in all enzyme systems where cozymase was involved. I had the opportunity of testing the influence of the coenzyme factor prepared from heart muscle and kindly given to me by Drs Dewan & Green. The experiment has been described in their paper [1938, p. 633]; it shows a very pronounced acceleration of the reaction on the addition of the factor preparation from heart muscle to an acetone liver preparation of alcohol dehydrogenase.

In order to prove that the differences described above in the behaviour of the crude ammonium sulphate and the acetone preparations were due solely to their unequal contents of the coenzyme factor, the presence of the factor in the liver had to be shown. Liver mince was washed with tap water for 24 hr. until

Table III. *Stability of the liver alcohol preparations on storing and effect of the coenzyme factor*

Thunberg tubes with 40 mg. enzyme preparation, 0·5 mg. cozymase, 1 mg. methylene blue, 0·1 ml. 96% alcohol, 2 ml. phosphate buffer pH ~7·6. 1 ml. coenzyme factor preparation (sheep liver).

Age of alcohol dehydrogenase preparation		Reduction time, min.	
		Ammonium sulphate	Acetone
Fresh preparation	Without factor	4 min.	58 min.
	With factor	4 min. 50 sec.	4 min. 30 sec.
2 weeks old	Without factor	4 min.	60 min.
	With factor	4 min. 50 sec.	4 min. 30 sec.
2 months old	Without factor	5 min. 30 sec.	3 hr.
	With factor	4 min.	4 min.
4 months old	Without factor	6 min.	Several hr.
	With factor	4 min.	6 min.

almost colourless, ground in water with sand, the coarse particles were removed by centrifuging or straining through muslin and the resulting uniform suspension was used. Alone it gave negative results when tested for alcohol dehydrogenase, aldehyde mutase and aldehyde dehydrogenase, but added to the acetone preparation of alcohol dehydrogenase it produced a very marked acceleration of the methylene blue reduction (Table III). Washed liver suspensions as used in these experiments retain their activity for several days when kept at 0°.

The coenzyme factor has been shown to be acetone-sensitive and thermolabile [Dewan & Green, 1937] but resistant to ammonium sulphate treatment [Euler & Hellström, 1938]. This explains the poor activity of the acetone preparations from the beginning and also their apparent continual deterioration on standing. From Table III it can be seen that when supplemented by the coenzyme factor there is no change in the activity of the acetone preparation during the whole period (4 months) of the experiment. In the ammonium sulphate preparation no acceleration was seen on addition of the factor. It is however enough to heat the liver extract for a short time to 52° before the ammonium sulphate precipitation (as described under (*b*) above), to obtain a preparation giving results similar to those of the acetone preparations.

Effects similar to those with the washed liver suspensions were also obtained with preparations of coenzyme factor made from skeletal muscle, spleen and brain tissue. Dewan & Green [1938] and Euler & Haasse [1938] state that the factor is present in a great variety of animal tissues.

In addition to the above results which seem to disprove completely the alleged instability of the alcohol dehydrogenase, I was never able to confirm statements regarding the sensitivity of the enzyme towards O_2 or on standing in solution. There was no significant loss of activity on aeration at 37° or on prolonged dialysis or in the solution after standing for 3–4 days.

Optimum pH. Reichel & Köhle [1935] state that the pH optimum of their preparations of liver alcohol dehydrogenase is 6·7–7·3. Using the methylene blue technique the optimum was found to be at pH 7·6, with a rapid fall below pH 7. The shift towards the alkaline side may be caused by the addition in these experiments of the coenzyme factor which is very acid-sensitive and comparatively resistant to alkali.

When however the bacterial alcohol oxidase was tested at various reactions from pH 4·5 to 8·3, the optimum was found at pH 5.

Estimation of the reaction product formed anaerobically

Data for anaerobic experiments on the alcohol dehydrogenase are given in Tables I and III. It remained however to estimate the product of the reaction with and without coenzyme factor.

In order to obtain enough material for a chemical determination samples 10 times larger than those normally required for ordinary Thunberg experiments were put up. Large (70 ml.) Thunberg tubes were found most convenient; their hollow stoppers were filled with M phosphoric acid to inactivate the enzyme at the end of the reaction, which was indicated by the reduction of the methylene blue. After efficient cooling in ice-water the fluid was quantitatively transferred to the distillation flask of the Friedemann *et al.* [1927] apparatus. The distillation into $NaHSO_3$ was continued for 15 min. and the estimation of acetaldehyde was carried out iodimetrically.

The actual composition of the various samples and controls together with the results obtained are given in Table IV.

Table IV. *Chemical estimations of acetaldehyde*

In Thunberg tubes (70 ml.) 5 ml. dialysed enzyme solution (=0·3 g. acetone preparation), 4 ml. phosphate buffer $M/20$, pH 7·6, 10 mg. methylene blue (MB), 5 mg. cozymase, 0·3 ml. 96% alcohol, 4 ml. factor preparation. In hollow stoppers 10 ml. M phosphoric acid.

In control experiments acid was added to the enzyme and factor before the other reagents.

1 ml. $M/100$ I_2 =0·22 mg. acetaldehyde.

1 mol. MB (355) corresponds to 1 mol. acetaldehyde (44).

Controls	Reduction time min.	ml. $M/100$ I_2
1. Enzyme + factor + cozymase + alcohol + MB	—	1·95
2. Factor (sheep liver, heart muscle)	—	0·10
3. Enzyme + factor (liver) + cozymase + MB + 1 mg. acetaldehyde	—	0·80

Experiments		
1. Enzyme + cozymase + alcohol + MB	80	4·45
2. Enzyme + cozymase + alcohol + heart muscle factor + MB	12	6·00
3. Enzyme + cozymase + alcohol + skeletal muscle factor + MB	16	6·60
4. Enzyme + cozymase + alcohol + liver factor + MB	8	2·20
5. Enzyme + cozymase + alcohol + liver factor + MB	10	2·35
6. Enzyme + cozymase + 1 mg. aldehyde + liver factor + MB, incubated for 10 min. at 37°	—	6·60

It can be seen from Table IV that acetaldehyde is produced in samples without additional coenzyme factor and also when a washed heart muscle suspension is added as the source of the factor. Acetaldehyde dinitrophenylhydrazone was also obtained in similar experiments by precipitation with 2:4-dinitrophenylhydrazine after removal of the proteins with trichloroacetic acid and adsorption of methylene blue on kaolin.

In all cases however where washed liver suspensions provided the coenzyme factor, acetaldehyde formation could not be detected. A control experiment (Table IV, Exp. 6) where, instead of alcohol, a known amount of acetaldehyde was incubated for 8 min. with the experimental mixture and recovered quantitatively, excluded the possibility of acetaldehyde disappearance due to dismutation or oxidation. No satisfactory explanation of this phenomenon could be found and it is not easy to see what could be the reaction product of alcohol dehydrogenation in this case.

Aerobic experiments on the alcohol dehydrogenase

Several preliminary experiments were first carried out on 1 : 1 aqueous liver extracts in which O_2 uptake was measured on addition of alcohol and cozymase. A very rapid and marked increase in O_2 uptake by the extracts was observed with alcohol and similarly, but to a smaller extent, with glycerol. It should however be pointed out that there was always a large "blank" O_2 uptake in the extracts which did not disappear even on very long autolysis and dialysis. The observed increase in O_2 uptake on addition of alcohol very likely represented some complicated reaction between alcohol (or its oxidation product) and the substrates already present in the extract.

When however the O_2 uptakes of the acetone or ammonium sulphate preparations of liver alcohol dehydrogenase were tested manometrically on addition of alcohol and cozymase, it was found that practically no O_2 was taken up unless a carrier was provided. Methylene blue was found to be a suitable carrier, pyocyanine acted equally well (Table V, Exps. 2–4), quinone in small concentrations was useless, in larger concentrations it inhibited the enzyme. Even in the presence of methylene blue the reaction still remained very slow. Some acceleration was evident when $M/6$ semicarbazide was added as acetaldehyde fixative (Table V, Exp. 7). Addition of suspensions of coenzyme factor from liver or skeletal muscle had no effect on the O_2 uptake; when however a washed heart muscle suspension was used a definite acceleration of the reaction was obtained (Table V, Exps. 8, 10). The explanation probably lies in the fact that when tested for O_2 uptake in presence of p-phenylenediamine, the heart muscle suspension showed a considerable activity due to cytochrome oxidase even without addition of cytochrome c, whereas the liver and skeletal muscle suspensions yielded negative results.

Table V. O_2 uptake of the liver alcohol dehydrogenase preparation

In Barcroft manometers. 30 mg. enzyme preparation, 5 mg. alcohol, 0·5 mg. cozymase, 0·1 and 1 mg. methylene blue (MB), 1 mg. pyocyanine hydrochloride, 2 mg. flavoprotein, 0·5 ml. $M/2$ semicarbazide, 1 ml. washed heart muscle or skeletal muscle preparation. Phosphate buffer $M/30$, pH 7·4, total volume 3·3 ml.

Enzyme + cozymase + alcohol with the addition of:	O_2 uptake μl./80 min.
1. —	20
2. 0·1 mg. MB	50
3. 1·0 mg. MB	120
4. 1·0 mg. pyocyanine	110
5. 2·0 mg. flavoprotein	30
6. 2·0 mg. flavoprotein + 1·0 mg. MB	90
7. $M/2$ semicarbazide	170
8. Washed heart muscle suspension	240
9. Washed skeletal muscle suspension	40
10. Washed heart muscle suspension	200

Chemical estimations of the reaction product in aerobic experiments were made, but gave unsatisfactory results owing mainly to the very prolonged reaction time (3–4 hr.) before theoretical values were reached. The presence of volatile acid (acetic acid) in these experiments especially with some of the more crude preparations explained the small yields of acetaldehyde. It pointed to the fact that acetaldehyde partly underwent either oxidation or dismutation to acetic acid.

Various alcohols. The activity of the liver alcohol dehydrogenase was tested in presence of other alcohols, mainly by the Thunberg technique. Propyl

alcohol gave values similar to ethyl alcohol; with methyl and amyl alcohols positive results were obtained. With saligenin, glycerol and α-glycerophosphate negative results were obtained even on addition of the coenzyme factor.

Table VI. *Various alcohols*

Thunberg tubes with 20 mg. enzyme preparation, 30 mg. substrate, 0·5 mg. cozymase, 1 mg. methylene blue, 1 ml. coenzyme factor, 2 ml. phosphate buffer pH 7·6.

		Reduction time
1.	Ethyl alcohol	1 min. 35 sec.
2.	Propyl alcohol	2 min.
3.	Amyl alcohol	5 min. 35 sec.
4.	Methyl alcohol	11 min.
5.	Saligenin	∞
6.	Glycerol	∞
7.	α-Glycerophosphate	∞

The comparatively slow oxidation of methyl alcohol (Table VI, Exp. 4) suggested the possibility of a poisoning of the enzyme by $H.CHO$ as the reaction product. But it was found that $H.CHO$ in small concentrations has little effect on the liver alcohol dehydrogenase and incidentally it was discovered that it undergoes in the liver preparations a typical dismutation to CH_3OH and $H.COOH$. Large quantities of $H.CHO$ (and also of $CH_3.CHO$) produce a definite inhibition of the alcohol dehydrogenase which is partly reversible after removal of the $H.CHO$ by dialysis.

The presence of methyl alcohol has no inhibitory effect on the oxidation of C_2H_5OH in liver enzyme preparations. In this respect the bacterial alcohol oxidase shows an interesting behaviour. It hardly oxidizes CH_3OH at all, but in the presence of CH_3OH the oxidation of C_2H_5OH is almost completely abolished. That this is due to a specific influence of the CH_3OH on the alcohol enzyme and not to a general poisoning effect on the bacteria was shown by the fact that other enzymes (aldehyde and/or glucose oxidation) of these acetic bacteria were found to be entirely unaffected by CH_3OH.

Inhibitors

Dixon [1937] has found that $M/1000$ iodoacetate completely inhibits the yeast alcohol dehydrogenase. However, negative results or only slight inhibition was obtained with $M/100$ iodoacetate on the animal alcohol dehydrogenase (Table VII; Table VIII, Exps. 3–6). It seemed desirable to examine more closely this difference in the behaviour of the yeast and liver enzymes.

It was thought that the negative results with the liver enzyme preparation might be due to the high content of combined and free SH-groups (glutathione) of the proteins and consequently to a complete decomposition of the iodoacetate during the incubation with the liver enzyme solution. To test this possibility the following procedure was chosen. The yeast alcohol dehydrogenase was prepared by the method used by Dixon [1937] and found to be completely inhibited by $M/100$ iodoacetate. Then varying amounts of the liver enzyme preparation, some of them after exhaustive dialysis and also in some cases after heat-inactivation, were incubated with iodoacetate (final concentration $M/20$) for 5–10 min. at 37°. Then they were.mixed with the yeast enzyme and the mixture was again incubated for some time at 37°. The activity was then tested by means of the methylene blue technique in the usual way. It was found that the inhibitory effect of iodoacetate upon the yeast enzyme was almost completely abolished in samples where comparatively large quantities of the

Table VII. *Effect of iodoacetate on the liver alcohol dehydrogenase*

30 mg. enzyme preparation, 0·5 mg. cozymase, 0·1 mg. alcohol 96%, 1 mg. methylene blue, 1 ml. factor preparation, iodoacetate (IAA) to give $M/50$ or $M/100$ final concentration. The enzyme solution was incubated with iodoacetate for 15 min. at 37° before the actual experiment.

	Reduction time
A. Ammonium sulphate preparation	
1. No IAA	6 min.
2. + IAA $M/100$	7 min.
3. + IAA $M/50$	7 min. 50 sec.
B. Acetone preparation	
Not dialysed:	
1. No IAA (without "factor")	28 min.
2. + IAA $M/100$ (without "factor")	29 min.
Dialysed:	
3. No IAA (without "factor")	30 min.
4. + IAA $M/100$ (without "factor")	36 min.
5. No IAA (with "factor")	3 min. 10 sec.
6. + IAA $M/100$ (with "factor")	5 min. 50 sec.

liver preparation, boiled or unboiled, were incubated with iodoacetate (Table VIII, B, Exps. 1, 3). If, however, small amounts of the liver enzyme were used, or after a very prolonged dialysis, the decomposition of iodoacetate by the SH-groups of the liver enzyme was comparatively small and there was enough iodoacetate left to exert its usual poisoning effect upon the yeast alcohol dehydrogenase (Table VIII, B, Exps. 2, 4). The liver enzyme nevertheless remained uninhibited (Table VIII, A, Exps. 4, 6).

Table VIII. *Effect of iodoacetate in mixtures of yeast—and liver—alcohol dehydrogenase preparations*

Thunberg tubes. 3% enzyme solutions in phosphate buffer $M/50$, pH 7·4; 0·5 mg. cozymase, 1 ml. $M/2000$ MB.

	ml. yeast enzyme	ml. liver enzyme	Final concentration of IAA	Reduction time
A. 1.	1·00	—	—	3 min. 35 sec.
2.	1·00	—	$M/100$	∞
3.	—	0·50, not dialysed	—	18 min.
4.	—	0·50, not dialysed	$M/100$	20 min.
5.	—	0·50, dialysed	—	25 min.
6.	—	0·50, dialysed	$M/100$	33 min.
7.	1·00	0·50, not dialysed	—	3 min. 50 sec.
8.	1·00	0·10, not dialysed	—	3 min.

B. Samples of liver enzyme + IAA (final concentration $M/50$) were incubated for 10 min. at 37°. 1 ml. of yeast enzyme solution was added and the mixture again incubated for 10 min. at 37° before testing with MB.

Yeast enzyme added to:	Reduction time
1. 0·50 ml. liver enzyme (not dialysed) + IAA	5 min. 45 sec.
2. 0·10 ml. liver enzyme (not dialysed) + IAA	60 min.
3. 0·50 ml. liver enzyme (boiled, not dialysed) + IAA	7 min. 30 sec.
4. 0·50 ml. liver enzyme (dialysed) + IAA	50 min.

Yet another experiment was done to prove that the liver alcohol dehydrogenase is resistant to iodoacetate. In a liver preparation which contained both the alcohol dehydrogenase and the aldehyde mutase, $M/80$ iodoacetate inhibited the mutase almost completely and had only a very small effect on the dehydrogenase.

The bacterial alcohol oxidase tested aerobically gave similarly negative results after prolonged incubation with iodoacetate. In anaerobic experiments a slight but reproducible inhibition was recorded.

A few other substances known to act as inhibitors in various enzyme systems were tested and their actions both on the liver alcohol dehydrogenase and aldehyde mutase compared. Urethane, oxalate, maleate and pyrophosphate produce a fairly large inhibition of the alcohol dehydrogenase (30–50 %) but none of the mutase. Both enzymes are cyanide stable. Quinine, morphine and nicotine in low concentration act as inhibitors of the mutase but have no effect on the alcohol dehydrogenase.

In experiments on the aldehyde mutase which will be shortly described in detail, it was found that ammonium sulphate inhibits and also partly destroys irreversibly the aldehyde mutase but leaves the alcohol dehydrogenase unaffected.

Methylene blue in concentrations suitable as a carrier for the alcohol dehydrogenase slows down considerably the mutase activity.

Animal tissues other than liver

When tested by the methods described in this paper, positive results were obtained with alcohol dehydrogenase preparations from intestine and kidney, but brain and muscle gave negative results.

DISCUSSION

The mechanism of the oxidation of alcohol in yeast has been much studied and recently Negelein & Wulff [1937] in Warburg's laboratory have isolated in crystalline form the alcohol dehydrogenase from yeast. However, the alcohol dehydrogenase of animal tissues has received much less attention in spite of its occurrence in various animal tissues. From the experimental evidence given in this paper several conclusions can be drawn regarding the properties and the behaviour of the animal alcohol dehydrogenase under various conditions.

It has been shown that the enzyme is able to oxidize alcohol both anaerobically and aerobically but it requires for its activity the presence of some additional substances. The coenzyme necessary for this reaction is cozymase, and this property seems to be general for all enzyme systems concerned with alcohol oxidation (yeast, animal tissues, bacteria and also probably plants).

Acetone-treated enzyme preparations require for their completion under anaerobic conditions the addition of the coenzyme factor, so that this factor must be regarded as an essential part of the enzyme system. The omission of the coenzyme factor led in older observations to the unjustified view that the enzyme is particularly unstable.

In tissue slices [Leloir & Munoz, 1938] or in liver extracts alcohol undergoes rapid oxidation, but the acetone- or ammonium sulphate-treated enzyme preparations react with O_2 slowly and only if a carrier such as methylene blue is provided. The fact that an acceleration of O_2 uptake can be achieved through heart muscle cytochrome oxidase suggests the probability of a similar process actually taking place when alcohol is oxidized in the intact liver tissue. It also explains why it has been frequently assumed that the alcohol oxidase system from animal tissues is a CN-sensitive system. We found that under anaerobic conditions cyanide either has no effect upon the enzyme or slightly accelerates the reaction, probably by fixing the aldehyde. Thus the cyanide inhibition observed is due solely to the poisoning of the cyanide-sensitive cytochrome oxidase system.

It is difficult to regard flavoprotein as a part of the animal alcohol dehydrogenase system. It has only a very small effect on the course of the reaction, when added to preparations repeatedly treated with acetone.

When alcohol is oxidized anaerobically by the enzyme preparation either alone or with the addition of a washed muscle suspension as coenzyme factor, acetaldehyde is found as the reaction product. It is difficult to account for the fact that no acetaldehyde is found when washed liver suspensions are used as the source of the coenzyme factor. Such washed liver suspensions alone show no aldehyde dehydrogenase or mutase activity, and when acetaldehyde is added and incubated with the experimental mixture composed of the liver enzyme preparation, liver suspension (coenzyme factor), cozymase and methylene blue, it is recovered quantitatively. The possibility remains of the alcohol dehydrogenation being directed into a hitherto unknown route in presence of the washed liver suspension, or of its being linked with some secondary reaction. But to prove this assumption purification and closer analysis of the liver suspension is necessary.

In experiments in which the course of the reaction is very slow, e.g. in aerobic conditions or anaerobically without additional coenzyme factor, the yield of acetaldehyde is smaller than would be expected from the amount of alcohol added and some acetic acid is produced. This can be accounted for by the presence in the preparations of aldehyde mutase and in some of the more crude preparations also of aldehyde oxidase. It explains why in the intact liver tissue acetic acid is found almost exclusively as the product of alcohol oxidation.

In contrast to the yeast alcohol dehydrogenase, the animal (and also the bacterial) alcohol dehydrogenase seems to be only little affected by iodoacetate, even if large concentrations of the iodoacetate are allowed to act upon the enzyme for some time. This finding, together with the fact that flavoprotein does not seem to be a constituent of the animal enzyme, suggests the possibility of some essential difference between the structures of the yeast and the animal alcohol dehydrogenases.

Dixon & Lutwak-Mann [1937] have shown that by suitable methods of preparation and purification it is possible to obtain the liver aldehyde mutase free from alcohol dehydrogenase. The present results, where the actions of various inhibitors were tested both on the alcohol dehydrogenase and on the mutase, furnish further evidence that these two enzymes are distinct systems.

SUMMARY

Various methods of preparation of the liver alcohol dehydrogenase and its properties are described.

By using suitable preparations it can be shown that cozymase and cozymase factor are essential components of the system.

According to the treatment, preparations can be obtained which contain no cozymase but have enough coenzyme factor (precipitation with ammonium sulphate) and vice versa (precipitation with acetone). The alcohol dehydrogenase in the preparations remains unaltered over a period of months.

Under certain experimental conditions acetaldehyde is obtained quantitatively as the reaction product of alcohol oxidation. The probability of other courses of alcohol oxidation is discussed.

The alcohol dehydrogenase in the preparations cannot react directly with O_2. The presence of carriers (methylene blue, pyocyanine) is necessary. The reaction with O_2 is markedly accelerated by addition of cytochrome oxidase preparations.

Ethyl, propyl, methyl and amyl alcohols are oxidized by the liver alcohol dehydrogenase preparations. With glycerol, α-glycerophosphate and. saligenin negative results are obtained.

Iodoacetate has little effect upon the animal alcohol dehydrogenase. The enzyme is not sensitive to cyanide. Evidence is provided for the non-identity of the liver alcohol dehydrogenase and aldehyde mutase.

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CLXXXII. THE MOLECULAR WEIGHT OF CROTOXIN

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SLOTTA & FRAENKEL-CONRAT [1938, 1] have succeeded in purifying and crystallizing the active substance in the venom from the rattlesnake (*Crotalus terrificus*). They showed that this snake poison is a protein, with a large sulphur content (4 %), and gave it the name crotoxin. As far as we know, this is the first snake poison ever isolated in a pure crystalline state. It was therefore of great interest to determine its molecular weight.

From analytical determinations of the methionine content of the protein molecule, Slotta & Forster [1938] conclude that the minimum molecular weight is 11,000. They state that it is very probable that the real size is three or six times this value.

We have determined the molecular weight of crotoxin by the ultracentrifugal methods developed in this laboratory as described by Svedberg [1937].

The partial specific volume of the crotoxin was found to be 0·704, which is a comparatively low value for a protein. Due to lack of material we were not able to make more than one determination (1 % protein in 4 % salt solution). However, the error should not exceed 2 %.

The sedimentation constant was determined in the ultracentrifuge at a speed of 70,000 r.p.m., corresponding to a centrifugal force of 350,000 *g*. The observations were made by the scale method of Lamm [1937]. All the solutions were prepared immediately before the experiments from a stock solution, containing 1 % crotoxin in 0·67 *M* NaCl. Table I gives the results.

Table I. *Sedimentation velocity determinations*

Crotoxin concentration %	Salts in solvent	Total salt molarity	pH	s_{20}*
1·0	NaCl	0·67	Not buffered	3·10
0·5	,,	0·67	,,	3·18
0·3	,,	0·2	,,	3·09
0·2	,,	0·2	,,	3·14
0·3	NaCl, HOAc	0·25	3·8	2·92
0·3	,,	0·25	3·8	2·95
0·3	NaCl, HOAc, NaOAc	0·25	4·4	2·92
0·3	,,	0·26	5·5	3·06
0·3	NaCl, KH_2PO_4, $Na_2B_4O_7$	0·23	8·7	3·21
0·3	NaCl, $Na_2B_4O_7$, Na_2CO_3	0·25	10·0	3·20

* Sedimentation constants are given in units of 10^{-13}.

The sedimentation constant is, within experimental error, independent of concentration. The average of the first four determinations is $s_{20} = 3\cdot13$. In acid solutions the sedimentation constant is a little lower, but in neutral and alkaline solutions it has about the same value. The sedimentation curves obtained are symmetrical and suggest a homogeneous substance.

The diffusion of crotoxin was measured according to Lamm's method [1937; see also Lamm & Polson, 1936]. The following results were obtained:

Table II. *Diffusion determinations*

Crotoxin concentration %	Salts in solvent	Total salt molarity	D_{20}*
1·0	NaCl	0·67	8·70
0·6	NaCl, Na_2HPO_4, KH_2PO_4	0·72	8·51
0·5	NaCl	0·67	8·55
0·25	NaCl	0·67	7·55

* Diffusion constants are given in units of 10^{-7}.

The average of the first three values is $D_{20} = 8·59$. The last value is probably not so accurate, owing to the low concentration. The diffusion curves are in all experiments very nearly normal distribution curves, indicating a homogeneous substance. The diffusion constant was calculated also from the spreading of the boundary in the first of the sedimentation experiments, which gave $D_{20} = 9·0$. Such a calculation cannot give the same accuracy as a static diffusion experiment, but its good agreement with the figures in Table II shows that crotoxin is a monodisperse protein.

Using the formula

$$M = \frac{RT\, s_{20}}{(1 - V\rho)D_{20}}$$

a value of 30,000 is obtained for the molecular weight of crotoxin. The ratio of the molecular frictional constant to that of a spherical molecule of the same weight, f/f_0, is 1·2. Slotta's value of 11,000 for the minimum molecular weight is, within experimental errors, one-third of our value.

To check the value we have also made a sedimentation equilibrium determination. The formula used for calculation is

$$M = \frac{2RT \ln (c_2/c_1)}{(1 - V\rho)\, \omega^2 (x_2^2 - x_1^2)}.$$

The speed of the centrifuge was 10,000 r.p.m. Two cells were used, placed at 5·0 and 5·7 cm. distance from the centre of rotation. They both gave the same average value of 30,500 for the molecular weight. There was no drift in the calculated values along the radius, showing that the substance is homogeneous. This is in agreement with the results of the diffusion measurements given above.

Slotta & Fraenkel-Conrat [1938, 2] have found that cysteine reduces the poison value (for definition see Slotta & Szyszka [1938]) of the crotoxin, probably due to its specific reduction power for protein-S—S-bonds. It was of interest to see whether it was possible to determine the particle size of the decomposition products. We therefore made an experiment according to Slotta's & Fraenkel-Conrat's experimental data. The only difference was that for ultracentrifugal determinations we needed a higher concentration of the protein than they used. We started with a 0·25 % solution of crotoxin; on standing with cysteine a precipitate was formed, which was probably cystine. The solution remaining was ultracentrifuged, and was found to contain very little high-molecular material, but consisted mainly of low-molecular products of variable, undeterminable size.

Crotoxin, which had been standing 3 days at 37° and pH 8·7, contained the usual high-molecular component (s_{20} was measured in two experiments, 3·39 and 3·08), but in addition decomposition products of low molecular weight were

present. It is of interest to note that Slotta has found a decrease in the poison value by this treatment.

It is therefore obvious that the poison activity is associated with the unchanged protein, as already pointed out by Slotta, and the decomposition products have no, or only a very small, activity, as is observed also with the decomposition products of enzymes and other active proteins.

SUMMARY

1. The partial specific volume of crotoxin was determined to be $V = 0.704$.

2. The sedimentation constant of crotoxin was found to be independent of concentration and of pH within the range 5·5–10·0. Its value was 3·13.

3. The diffusion constant was found to be 8·59.

4. Crotoxin behaved in sedimentation and diffusion as a homogeneous substance, and the molecular weight was calculated to be 30,000. The frictional ratio is 1·2.

5. Sedimentation equilibrium measurements gave the value 30,500 for the molecular weight, in good agreement with the preceding value.

6. The molecule is split into smaller, inactive substances by treatment with cysteine or by warming in alkaline solution.

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CLXXXIII. THE $l(+)$GLUTAMIC DEHYDRO-GENASE OF ANIMAL TISSUES

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THUNBERG [1920] observed that natural glutamic acid was oxidized in the presence of frog muscle. Weil-Malherbe [1936] was the first to prepare an enzyme extract which catalysed this oxidation but he did not resolve the system into its catalytic components.

While this present analysis of the $l(+)$glutamic dehydrogenase system was in progress Adler *et al.* [1937] described the properties of the same enzyme in liver. They found that coenzyme I was essential to the system and that the products of the reaction were α-ketoglutaric acid and ammonia. They also demonstrated the reversibility of the reaction.

I. *Preparation of the components*

The $l(+)$glutamic dehydrogenase was prepared from liver or kidney of pig as follows. About 300 g. liver or kidney are minced in a Latapie mincer. To the mince are added 4 vol. cold acetone and the mixture is stirred for about 3 min., filtered on a Büchner funnel and washed in turn with acetone and ether. The material on the filter paper is again mixed with 4 vol. acetone, filtered, washed with acetone and ether and spread out on paper to dry in a current of air. 10 g. of the dry powder are rubbed up with 100 ml. distilled water and the mixture centrifuged. The supernatant fluid after filtration is acidified with 10 % acetic acid to pH 4·6, the centrifuged precipitate washed with distilled water and finally suspended in 25 ml. $M/10$ phosphate buffer, pH 7·3. The enzyme keeps its activity in solution at 0° for 4–5 days. The acetone powder can be kept *in vacuo* for several weeks without loss of activity.

Coenzyme I was prepared by the method outlined by Green *et al.* [1937].

II. *The reaction with molecular oxygen*

A mixture of the dehydrogenase, coenzyme I and $l(+)$glutamic acid in the presence of a carrier such as pyocyanine takes up O_2 readily. The rate of O_2 uptake is dependent on the concentrations of the various reactants, i.e. dehydrogenase, coenzyme, substrate and carrier (cf. Figs. 1–4). As in other coenzyme systems the proportionality between the rate of oxidation and the concentrations of the different components holds only at low concentrations. Above some limiting concentration the system becomes saturated with the component in question.

The Michaelis constant, i.e. the substrate concentration at which half the maximum velocity is reached is approximately $M/133$ (Fig. 3).

Carriers which have been found to be active in the $l(+)$glutamic acid system include pyocyanine, methylene blue, cytochromes a and b, flavinphosphate and flavoprotein from yeast (Table I). Cytochrome c in large concentrations (1·2 mg./ml.) has a small effect on the O_2 uptake. The most efficient carrier
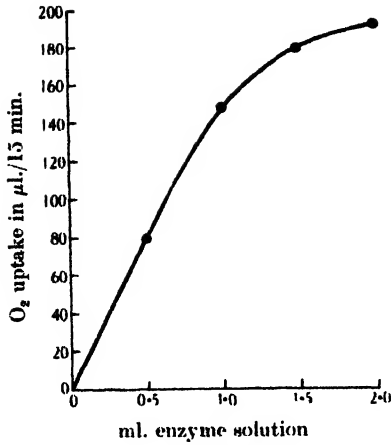
Fig. 1.
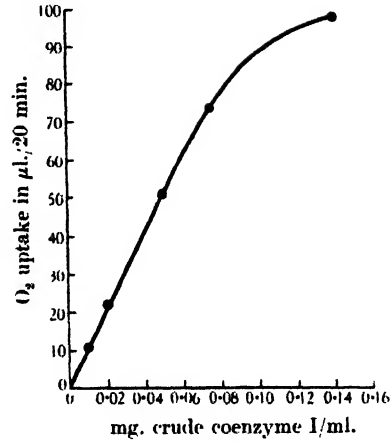
Fig. 2.

Fig. 1. The effect of the concentration of dehydrogenase. Each manometer contained 1·0 ml. 0·3% coenzyme 1, 0·2 ml. 0·1% pyocyanine and 0·5 ml. $M/3$ $l(+)$glutamic acid.

Fig. 2. The effect of the concentration of coenzyme I. Each manometer contained 1·5 ml. enzyme, 0·2 ml. 0·1% pyocyanine and 0·5 ml. $M/3$ $l(+)$glutamic acid.
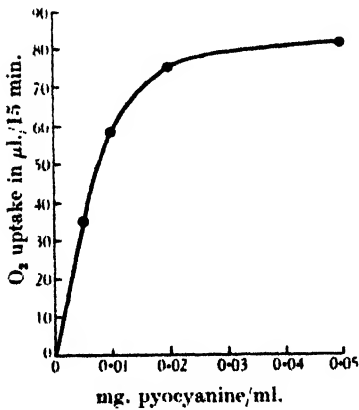
Fig. 3.
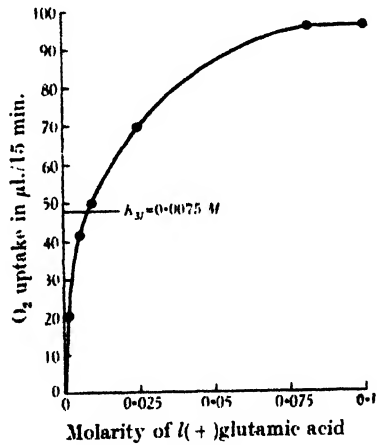
Fig. 4.

Fig. 3. The effect of the concentration of pyocyanine. Each manometer contained 1·5 ml. enzyme, 1·0 ml. coenzyme 1 and 0·5 ml. $M/3$ $l(+)$glutamic acid.

Fig. 4. The effect of the concentration of substrate. Each manometer contained 1·0 ml. enzyme, 1·0 ml. 0·3% coenzyme I and 0·2 ml. 0·1% pyocyanine. The total volume was 3·3 ml.

Table I. *The effect of carriers on the reaction with O_2*

The system contained 1·5 ml. enzyme; 1·0 ml. 0·3% coenzyme and 0·5 ml. $M/3$ $l(+)$glutamic acid. The total volume was 3·3 ml.

	$\mu l.$ O_2 in 15 min.
$l(+)$glutamic system without carrier	0
System + 1·0 mg. methylene blue	56
System + 0·2 mg. pyocyanine	84
System + 0·5 mg. flavinphosphate	24
System + 2·5 mg. pure flavoprotein (yeast)	56
System + 0·3 ml. cytochromes a and b preparation	125

system was the cytochrome a and b system of Keilin and Hartree (cf. Dewan & Green [1938] for method of preparation). It has already been shown in other coenzyme systems that the coenzyme factor rapidly catalyses the oxidation of reduced coenzyme by cytochromes a and b [Dewan & Green, 1938].

$M/100$ cyanide inhibited the $l(+)$glutamic system when the cytochromes were used as carriers (Table II). The inhibition was practically complete during the first 5 min. but became progressively less as the reaction proceeded owing to the accumulation of α-ketoglutaric acid and subsequent cyanohydrin formation. $M/500$ cyanide reduced the rate of O_2 uptake by about 66 % in the first 5 min. Here again the extent of inhibition decreased as the reaction progressed. Cyanide even in $M/10$ concentration had no inhibitory effect when pyocyanine was used as carrier.

Table II. *The effect of cyanide on the* $l(+)$*glutamic system with cytochromes* a *and* b *as carriers*

The complete system contained 1·5 ml. enzyme, 1·0 ml. 0·3 % coenzyme I, 0·5 ml. preparation of cytochromes a and b and 0·3 ml. $M/3$ $l(+)$glutamic acid.

	μl. O₂ in 5 min.	μl. O₂ in 10 min.
Complete $l(+)$glutamic system	30	53
With $M/500$ HCN	11	25
With $M/100$ HCN	0	5

Ketone fixatives such as hydrazine, semicarbazide and cyanide were tested for their effect on the velocity of oxidation of $l(+)$glutamic acid. Hydrazine had no appreciable effect. Semicarbazide and cyanide did not influence the

Table III. *The effect of fixatives*

The system contained 1·5 ml. enzyme, 1·0 ml. coenzyme, 0·2 ml. 0·1 % pyocyanine, 0·4 ml. $M/3$ $l(+)$glutamic acid. The fixative solutions were neutralized.

	μl. O₂		
	10 min.	20 min.	30 min.
$l(+)$Glutamic system	142	200	226
+0·2 ml. $M/2$ semicarbazide	157	222	260
+0·2 ml. $2M$ HCN	162	246	300
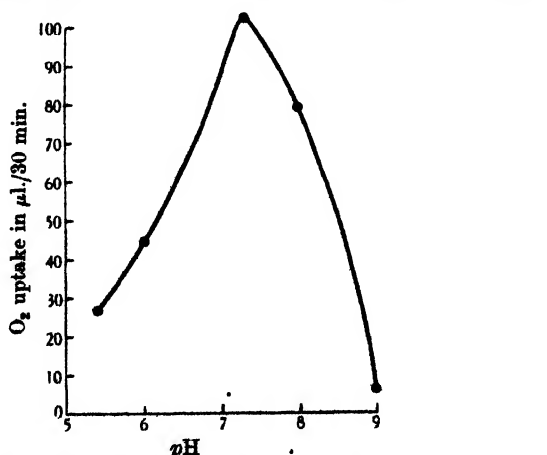
Fig. 5. The effect of pH. Each manometer contained 1·0 ml. enzyme, 0·8 ml. coenzyme, 0·2 ml. 0·1 % pyocyanine and 0·3 ml. $M/3$ $l(+)$glutamic acid.

initial velocity but had a slight effect in preventing falling off in velocity as the reaction proceeded (cf. Table III). α-Ketoglutaric acid inhibits the oxidation of $l(+)$glutamic only when present in high concentration. This indicates that the glutamic system is much more negative in potential than the coenzyme system.

Fig. 5 shows the dependence of the rate of oxidation of $l(+)$glutamic acid on the pH of the solution. The maximum velocity is observed at about pH 7·3, the rate falling off rapidly above and below this value.

Effect of coenzyme factor

The presence of an enzyme in animal tissues which catalyses the oxidation of reduced coenzyme by carriers was demonstrated by Dewan & Green [1938] and Adler et al. [1937]. Coenzyme factor is also necessary in the $l(+)$glutamic system. To obtain a dehydrogenase preparation free from the factor, pig kidney, after mincing in a Latapie mincer, was allowed to stand 30 min. in 2 vol. iced water and pressed through muslin. The filtrate was mixed with 3 vol. cold acetone, the precipitate washed with acetone and ether and rubbed up in a mortar until dry. The acetone powder was then treated in the usual manner of preparing the $l(+)$glutamic dehydrogenase. The enzyme solution before using was filtered through kieselguhr and charcoal to remove last traces of coenzyme factor. The factor was prepared from rabbit skeletal muscle [cf. Dewan & Green, 1938].

Table IV shows that coenzyme factor is necessary for the aerobic oxidation of $l(+)$glutamic acid in presence of dehydrogenase, coenzyme I and pyocyanine.

Table IV. Effect of coenzyme factor

The complete system contained 0·5 ml. enzyme, 1·0 ml. coenzyme, 0·05 ml. 0·1 % pyocyanine, 1·0 ml. factor and 0·5 ml. $M/3$ $l(+)$glutamic acid.

System	μl. O_2 in 30 min.
System	31
Without factor	3
Without enzyme	0
Without glutamic acid	0

III. The products of oxidation

$l(+)$Glutamic acid on oxidation yields α-ketoglutaric acid and NH_3. The ketonic acid was isolated and identified as follows. A mixture containing 100 ml. enzyme, 50 ml. 0·3 % coenzyme I, 10 ml. 0·1 % pyocyanine and 25 ml. $M/3$ $l(+)$glutamic acid was aerated vigorously at 37° for 90 min., deproteinized with 50 % trichloroacetic acid and filtered. The filtrate was concentrated in vacuo to 25 ml. 1 g. 2:4-dinitrophenylhydrazine in 100ml. $2N$ HCl was added to the filtrate when crystals of the 2:4-dinitrophenylhydrazone began forming at once. The mixture was left at 0° for 10 hr. The precipitate was washed with $2N$ HCl and dried. Just sufficient ethyl acetate was added to dissolve the crystals and a small brown residue was filtered off. To the filtrate were added 4 vol. ligroin. The precipitate was centrifuged, washed with ligroin and finally dried in vacuo. M.P. 218°; M.P. of synthetic α-ketoglutaric acid 2:4-dinitrophenylhydrazone 218°; mixed M.P. 218°. Found (Weiler): C,40·81 %; H, 3·31 %; N, 17·1 %. $C_{11}H_{10}O_8N_4$ requires C, 40·44 %; H, 3·09 %; N, 17·18 %.

The ratio of α-ketoglutaric acid formed to O_2 absorbed was determined by estimation of the $NaHSO_3$-binding of the deproteinized system after recording the O_2 uptake. Table V shows that for every atom of oxygen absorbed, approximately 1 mol. α-ketoglutaric acid was formed.

Table V. *Estimation of α-ketoglutaric acid formed by oxidation of* l(+)*glutamic acid*

The complete system contained 1·5 ml. enzyme, 1·0 ml. coenzyme, 0·2 ml. 0·1 % pyocyanine and 0·5 ml. $M/3$ l(+)glutamic acid. Control had no substrate.

mg. α-ketoglutaric acid found by measuring $NaHSO_3$-binding power	2·2
μl. O_2 absorbed during the oxidation of the l(+)glutamic acid	172
Theoretical amount (in mg.) of α-ketoglutaric acid, assuming 1 mol. α-keto-glutaric acid is produced for each atom O absorbed	2·25

Further confirmation that 1 atom O is absorbed for each mol. l(+)glutamic acid oxidized is obtained from the O_2 equivalence of small amounts of l(+)-glutamic acid. Table VI shows the close agreement between the O_2 uptake for known amounts of l(+)glutamic acid and the theoretical uptake, calculated on this basis. With larger amounts of substrate the O_2 uptake was somewhat lower than the theoretical value, due to the fact that enzymic activity ceases before the whole of the glutamic acid is oxidized.

Table VI. *The O_2 equivalence of* l(+)*glutamic acid*

Each manometer contained 2·0 ml. enzyme, 1·0 ml. coenzyme I and 0·2 ml. 0·1 % pyocyanine. The substrate was placed in Keilin cups which were introduced into the main body of the fluid after equilibration. The control O_2 uptake without substrate has been subtracted from the experimental values.

Millimol. l(+)glutamic acid	0·25	0·5	0·75	1·0
μl. O_2 absorbed	26	58	81	94
Theoretical uptake assuming that 1 atom O reacts with 1 mol. glutamic acid	28	56	84	112

NH₃ estimations. The O_2 uptake of the complete glutamic system was measured manometrically. A control experiment containing the whole system with the exception of substrate was carried out simultaneously. After a run of 90 min. NH_3 was estimated by the Parnas method. Table VII shows that the amount of NH_3 found correlates well with the theoretical value calculated from the O_2 uptake.

Table VII. *NH₃ estimations*

Complete system contained 1·0 ml. enzyme, 1·0 ml. coenzyme, 0·2 ml. 0·1 % pyocyanine and 0·5 ml. $M/3$ l(+)glutamic acid.

mg. NH_3 found (corrected for control)	Theoretical NH_3 assuming that for 1 atom O absorbed 1 mol. NH_3 is produced	% theory
(1) 0·17	0·18	94
(2) 0·26	0·28	92

IV. *Specificity of substrate*

The glutamic dehydrogenase specifically catalyses the oxidation of l(+)-glutamic acid to α-ketoglutaric acid and NH_3. The unnatural isomeride is not oxidized.

dl-β-Hydroxyglutamic acid was attacked at one-tenth the rate of l(+)glutamic acid. Alanine, phenylalanine, valine, histidine and leucine of the l-series are not oxidized in the presence of the dehydrogenase preparation. Some preparations catalysed the oxidation of l-aspartic acid and l-cystine but these oxidations were found to be unconnected with the glutamic dehydrogenase. A sample of glutamine showed slight activity, but as it contained small amounts of glutamic acid it was difficult to assess the results.

V. *Specificity of coenzyme*

Coenzyme I cannot be replaced by coenzyme II in the $l(+)$glutamic system (cf. Table VIII). Coenzyme II was prepared from horse red blood cells and its activity checked with the hexosemonophosphate system.

Table VIII. *Specificity of coenzyme*

Experiments were carried out under anaerobic conditions in Thunberg tubes. The $l(+)$glutamic system contained 2 ml. enzyme, 0·2 ml. 0·5% methylene blue and 0·5 ml. of $M/3$ $l(+)$glutamic acid. The Thunberg tubes after evacuation were immersed in a water bath at 38 .

System	Reduction time of methylene blue min.
+ 0·15 mg. coenzyme I	∞
+ 0·15 mg. coenzyme I	5
+ 0·15 mg. coenzyme II	∞

VI. *Reduction of coenzyme I*

Coenzyme I was reduced by $l(+)$glutamic acid in the presence of the dehydrogenase (cf. Table IX). The reduction was demonstrated spectrophotometrically by the appearance of the characteristic band of reduced coenzyme with a peak at 340 mμ.

Table IX. *Reduction of coenzyme I by* $l(+)$*glutamic acid in presence of the dehydrogenase*

The system contained 1·0 ml. 0·3% oxidized coenzyme I, 0·3 ml. $l(+)$glutamic acid and 3·1 ml. 0·25% $NaHCO_3$. Total vol. 8 ml. Incubated in Thunberg tubes at 38" for 5 min. Contents then boiled and filtered through kieselguhr. The clear filtrate was used directly for analysis in the Hilger Spekker spectrophotometer [cf. Green & Dewan, 1937]. Two controls were done, one containing the system without $l(+)$glutamic acid, the other without the dehydrogenase.

System	$\log I_0/I$ at 340 mμ
System	0·7
System without $l(+)$glutamic acid	0·1
System without dehydrogenase	0·1
Oxidized coenzyme	0·1

VII. *Distribution*

Various tissues of pig were investigated for the presence of $l(+)$glutamic dehydrogenase. Acetone powders of the tissues were made in the usual way. 4 g. of the powder in each case were ground up in a mortar with 40 ml. distilled water, centrifuged, the supernatant liquid filtered from fat and precipitated with 10% acetic acid at pH 4·6. The sediment was washed with distilled water and resuspended in 10 ml. $M/10$ phosphate buffer, pH 7·3. The system contained 2 ml. enzyme, 1·0 ml. 0·3% coenzyme I, 0·2 ml. 0·1% pyocyanine and 0·5 ml. $M/3$ $l(+)$glutamic acid. A control containing the system without $l(+)$glutamic acid was carried out in each case and the O_2 uptake subtracted from the value obtained with the complete system.

	μl. O_2 in 30 min.
System using liver enzyme	274
System using kidney enzyme	270
System using heart enzyme	25

The enzyme was detected in muscle and brain but in concentrations too small for accurate measurements.

VIII. *Reversibility*

The reaction between glutamic acid and coenzyme I may be formulated as follows:

l(+)Glutamic acid + coenzyme I→α-ketoglutaric acid + NH₃ + reduced coenzyme I.

If this reaction is reversible it should be possible to obtain glutamic acid from a mixture of the reduced coenzyme I, NH₃ and α-ketoglutaric acid. Reduced coenzyme I can be obtained either enzymically or by reduction with hyposulphite. In the following procedure the β-hydroxybutyric system was used to reduce the coenzyme, and the production of acetoacetic acid under anaerobic conditions was used as a measure of the reaction between reduced coenzyme on the one hand, and NH₃ and α-ketoglutaric acid on the other. For the theory of this method, cf. Dewan & Green [1937] on coenzyme-linked reactions. Acetoacetic acid was measured by the aniline citrate method of Ostern [1933]. The enzyme was prepared from pig's heart [cf. Green *et al.* 1937]. This preparation contains dehydrogenases for both *l*-β-hydroxybutyric acid and *l*(+)glutamic acid. Table X shows that the reversed reaction takes place when all components of both systems are present. The small blank in the absence of NH₄Cl is due to the reversibility of the α-hydroxyglutaric dehydrogenase system which has been shown by Weil-Malherbe [1937] to be present in heart.

Table X. *Production of acetoacetic acid in the reaction between β-hydroxybutyric acid, α-ketoglutaric acid and* NH_3

The complete system contained 0·2 ml. *M dl*-β-hydroxybutyric acid, 0·8 ml. *M*/3 NH₄Cl, 0·2 ml. *M*/6 α-ketoglutaric acid, 1·5 ml. heart enzyme preparation and 1 ml. coenzyme 1. The substrates were neutralized before using.

	CO₂ production in 60 min.
Complete system	190
Without *dl*-β-hydroxybutyric acid	0
Without NH₄Cl	27
Without α-ketoglutaric acid	0
Without heart enzyme preparation	0
Without coenzyme I	0

Further proof of the reversibility of the *l*(+)glutamic system is derived from the demonstration of the formation of amino-N when all the components of the coenzyme-linked reaction were present. Three mixtures were set up in Thunberg tubes under anaerobic conditions; one contained the complete system, a second was without NH₄Cl and a third without α-ketoglutaric acid. After 1–2 hr. incubation at 38° the contents of each tube were deproteinized and the filtrates made up to the same final volumes. Acetoacetic acid estimations were made on one half of the filtrates and amino-N (Van Slyke method) on the other half.

Table XI. *Production of amino-N in the reaction between β-hydroxybutyric acid, α-ketoglutaric acid and* NH_3

Complete system contained 3·0 ml. heart enzyme preparation, 1·5 ml. 0·3 % coenzyme I, 0·2 ml. *dl*-hydroxybutyric acid, 1·0 ml. *M*/3 NH₄Cl and 0·2 ml. *M*/6 α-ketoglutaric acid. Two controls were done, one without NH₄Cl, the other without α-ketoglutaric acid.

	(1)	(2)
Amino-N found	0·04 mg.	0·068 mg.
Theoretical amount of amino-N expected from CO₂ production assuming that 1 atom amino-N is formed for each mol. CO₂ produced.	0·05 mg.	0·081 mg.

The small amino-N values in the controls were averaged and this value was subtracted from the amount obtained in the complete system. Table XI shows that the amino-N found agrees well with the theoretical amount expected from the acetoacetic acid production on the assumption that 1 atom amino-N is formed for each mol. acetoacetic acid produced.

SUMMARY

1. The preparation and properties of $l(+)$glutamic dehydrogenase of animal tissues are described. Coenzyme 1 is a necessary component of the system.

2. The dehydrogenase catalyses the oxidation of $l(+)$glutamic acid to α-ketoglutaric acid and NH_3. The reversibility of this reaction has been demonstrated.

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CLXXXIV. THE PURIFICATION OF URICASE

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IN the course of an attempt to isolate the enzyme uricase, which oxidizes uric acid to allantoin, it has been found possible to obtain a preparation 550 times purer than the liver powder which is used as starting material [Davidson, 1938]. The purest preparation contains 0·15–0·20 % Fe, a trace of Cu, no Co or Mn and 14·4 % N. Small quantities are colourless, and even very large quantities have only a very pale brown colour. It is therefore not a haemin derivative. It is quite insoluble in water, almost insoluble in phosphate buffer pH 7·4 but soluble in alkaline solution, e.g. borate buffer pH 10. Truszkowski [1934] and Ro [1931] have already shown that uricase is soluble in alkaline solution.

In order to determine the enzymic activity a manometric test has been developed. The main chamber of the manometer vessel contains 1·0 ml. $M/5$ borate buffer pH 9 and 1·5 ml. enzyme preparation plus water. The potash tube contains 0·2 ml. 10 % KOH, and the side bulb 2·24 mg. uric acid as $M/30$ lithium urate. (0·56 g. uric acid is dissolved in 35 ml. boiling $N/10$ lithium hydroxide and the solution is made up to 100 ml. with water. This solution must be freshly prepared each day.) The gas space contains pure oxygen and the oxygen uptake is measured at 38° over a period of 30 min.

By the specific activity of the enzyme is meant the number of μl. O_2 which are taken up per min. in the first 30 min. by 1 mg. of dry enzyme preparation. The specific activity of the best preparation is 85–90 μl. per mg. per min. as compared with a value of 0·12–0·15 μl. per mg. per min. for the dry liver powder from which it is obtained.

The most convenient starting material has been found to be pig's liver, which has already been used by Kleinmann & Bork [1933] and by Keilin & Hartree [1936] for uricase studies. The pig's liver is finely minced and ground in a mortar with five parts of acetone. The solid material is centrifuged off and shaken with three parts of acetone. After centrifuging, the liver powder is dried at first rapidly in a current of air and then overnight in vacuum desiccators, and is then finely powdered and passed through a sieve. The resulting light brown powder has a specific activity of 0·12–0·15 μl. per mg. per min. and retains its activity for several months when stored in desiccators at room temperature. Samples showing a specific activity of less than 0·10 μl. per mg. per min. are discarded.

250 g. liver powder are stirred with 2·5 l. ice-cold $M/10$ phosphate buffer pH 7·4. After standing for 20 min. at 0° the mixture is centrifuged, and the extract, which contains much protein but only little uricase is discarded. The solid residue is then stirred with 5 l. $M/10$ borate buffer pH 10 at 38°. After 20 min. the mixture is rapidly cooled to 0° and centrifuged. The clear extract contains the bulk of the enzyme and has a specific activity of 0·5 μl. per mg. per min.

An equal volume of saturated ice-cold ammonium sulphate solution is added to this solution, and the resulting precipitate, which contains the enzyme, is centrifuged off at 0° and dissolved with shaking in 5 l. ice-cold distilled water.

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1/10 vol. saturated ice-cold ammonium sulphate is then added (no precipitate being formed), and the solution, which has a pH of $7\cdot2$–$7\cdot4$, is heated to 55° for 5 min. with vigorous stirring, whereby a heavy flocculent precipitate of denatured protein is formed. The solution is rapidly cooled to 0° and the precipitate is centrifuged off and discarded. The enzyme solution, which now has a specific activity of $1\cdot0$–$1\cdot5$ μl. per mg. per min., is treated with enough saturated ammonium sulphate solution at 0° to bring the degree of saturation to 0·5. The resulting precipitate, which contains the enzyme, is centrifuged off at 0° and dissolved in 1 l. of ice-cold distilled water. This solution is dialysed for 15 hr. in cellophane sacs against running distilled water at 0°. A brown precipitate which appears during the dialysis and which contains the enzyme is centrifuged off and washed with ice-cold water on the centrifuge. It is then rubbed in a cooled mortar with 400 ml. $M/10$ phosphate buffer pH 7·4 at 0° and centrifuged on the high speed centrifuge (15,000 r.p.m.). The extract, which contains much protein but little enzyme, is discarded. The residue is rubbed in a mortar with 250 ml. $M/10$ borate buffer pH 10 at room temperature and is centrifuged on the high speed centrifuge. The extraction with 250 ml. borate buffer is repeated, the extracts, which contain the bulk of the enzyme, are combined, and the residue, which consists of brown insoluble protein, is discarded.

The clear, pale yellow uricase solution which now has a specific activity of 10–25 μl. per mg. per min. is cooled to 0°, treated with an equal volume of saturated ice-cold ammonium sulphate solution, and the resulting precipitate, which contains the enzyme, is centrifuged off at 0° and dissolved in 60 ml. ice-cold water.

To the solution just enough (0·5–1·0 vol.) ice-cold 80 % acetone is added so that a flocculent precipitate appears after 20 min. The precipitate is centrifuged off and discarded. Acetone is removed from the supernatant fluid by vacuum distillation at 20° with two drops octyl alcohol and the resulting slightly turbid solution, after standing overnight at 0°, is centrifuged on the high speed centrifuge. A small precipitate of very high activity is thus obtained, and is washed three times on the centrifuge with ice-cold distilled water. It is then rubbed up with 5 ml. $M/10$ borate buffer pH 10 at room temperature, in which the enzyme dissolves, leaving a small inactive brownish residue which is centrifuged off at high speed and discarded.

The uricase solution is almost colourless. When it is dialysed against running distilled water at 0° the free enzyme is obtained. Specific activity 85–90 μl. per mg. per min. The yield of the purest preparation is about 5 mg. per 100 g. liver powder.

On standing the activity of the free enzyme gradually diminishes but in solution at pH 10 it is stable for several weeks. Some of the enzyme frequently precipitates out in an insoluble form. The enzyme cannot be dried without great loss of activity.

The velocity of the test reaction is dependent on the oxygen pressure and in a mixture of 2 % oxygen and 98 % argon it is only 7 % as great as 100 % oxygen. If the argon is replaced by carbon monoxide no inhibition by the carbon monoxide is found.

Very small quantities of cyanide, however, inhibit the reaction reversibly. as has been already pointed out by Keilin & Hartree [1936] and by Truszkowski [1930]. The enzyme therefore would appear to be a heavy metal compound, but it has nevertheless not yet been proved that the catalytic activity of the enzyme is due to the iron. Attempts to remove iron by dialysis with cyanide after the manner in which Kubowitz [1938] removed copper from phenoloxidase, and by

other methods, have not so far been successful. The colourless nature of the enzyme suggests that the iron is not present in the form of a haemin derivative. On the other hand the comparatively large amount of iron (of the same order as that found by Sumner & Dounce [1937] in crystalline catalase) and the consistency with which the value 0·15–0·20% is found in repeated preparations, suggests that the iron is more than a mere impurity.

If it is assumed that the catalytic activity of the enzyme is due to the iron, then 1 mg. iron brings about the reaction of 57,000 μl. oxygen per min.

SUMMARY

A method for the purification of the enzyme uricase is described.

The purest preparation has a specific activity, under stated conditions, of 85–90 μl. per mg. per min. and contains 0·15–0·20% Fe.

Although uricase seems to be a heavy metal compound it has not yet been proved that the iron acts as the active group of the enzyme.

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CLXXXV. GLYCOLYSIS OF TRIOSE DERIVATIVES BY EXTRACTS OF TUMOUR AND OF MUSCLE[1]

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IN previous papers [Scharles *et al.* 1935; 1, 2] it was shown that tumour extracts can produce considerable amounts of lactic acid from hexosephosphates. The behaviour of tumour extracts was found to differ from that of muscle extracts in several features, and it was concluded that "...carbohydrate breakdown in the two tissue extracts proceeds along different paths" [1935, 1]. Boyland & Boyland [1935], however, working also with hexosediphosphate arrived at the opposite conclusion, namely that "the rapid breakdown of hexosediphosphate by tumour extracts along the same paths as occur in muscle extracts gives support to the idea that the mechanisms of lactic acid formation in tumour and muscle are similar"; and they have maintained this position in subsequent publications [Boyland *et al.* 1937; Boyland & Boyland, 1938].

It is the purpose of this paper to report two types of experiment which demonstrate that glycolysis in muscle and tumour extracts follows different pathways.

EXPERIMENTAL

The experiments reported here consisted in a comparison of lactic acid formation by muscle and tumour extracts. As a preliminary step substrates were prepared, first, by the action of NaF-poisoned tissue extracts on glycogen in phosphate buffers. Secondly, hexosediphosphate and pyruvic acid were likewise used as substrates.

Methods. The methods and procedures used here, unless otherwise stated, were the same as those described previously [Scharles *et al.* 1935, 1].

Fluoride substrates. It has been known for many years that NaF prevents the production of lactic acid in muscle brei, although glycogen continues to disappear [Embden & Deuticke, 1934]. The carbohydrate which disappears is accounted for, in part at least, by the accumulation of phosphate esters as described by several authors [Embden & Zimmermann, 1924; Lohmann, 1930; Embden *et al.* 1933]. In such experiments these esters have been regarded as intermediates in the formation of lactic acid from glycogen. They are believed to accumulate as the result of a more or less specific inhibition of the reactions by which they normally produce lactic acid, with less or no inhibition of the reactions leading to their formation [Embden *et al.* 1933]. It was important, therefore, to determine whether any differences could be detected between tumour and muscle extracts in regard to the nature of the products formed

under these experimental conditions. Accordingly, glycogen solution in phosphate buffer at pH 7·0 was incubated with muscle or tumour extract in the presence of NaF. After removal of the protein with trichloroacetic acid and of the inorganic P with Ba, the P esters were then isolated by precipitation of the Ba salts from alkaline alcoholic solution.

The data of Table I contain most of the experimental details of these experiments. The method of preparing the tissue extract, and other experimental and

Table I. *Preparation of NaF substrates*

				Phosphate			
Tissue	No.	NaF concentration M	Time of incubation hr.	Initial mg. P/ 100 ml.	Final mg. P/ 100 ml.	Esterified mg. P/ 100 ml.	Isolated mg. P/ 100 ml.
Rat muscle	M 4	0·005	4	398	287	111	41
,,	M 5	0·005	8	308	179	178	63
,,	M 6	0·005	4	102	62	40	21
,,	M 7	0·005	8	276	107	169	72
Mouse sarcoma 180	T 3	0·0075	8	124	52	72	35
,,	T 4	0·010	4	406	327	79	27
,,	T 5	0·0055	8	198	101	97	44
,,	T 6	0·005	8	327	210	117	44

analytical procedures were the same as those used previously [Scharles *et al.* 1935, 2]. The procedure for the isolation of the Ba salts was as follows.

The trichloroacetic acid filtrate (c. 5% CCl_3CO_2H) was neutralized in the cold with solid $Ba(OH)_2$, or a saturated solution and 2 g. $BaCl_2$ were added for each 100 ml. solution. The solution was then filtered to remove the precipitated $Ba_3(PO_4)_2$. The esters were isolated from the filtrate by the addition of 3 vol. alcohol. In some cases the esters were separated into two fractions, one precipitated at c. pH 7 and the other after making alkaline to thymol blue with NaOH. In other experiments all the esters were precipitated in one operation at about pH 8. Only 50% or less of the esterified P was recovered. The losses were found to be the result of inclusion of organic P in the $Ba_3(PO_4)_2$ precipitate, but the usual methods of recovering such losses by solution and re-precipitation did not greatly increase the yield. On the other hand, there was never any difficulty in recovering substantially all of the organic P of the $Ba_3(PO_4)_2$ filtrate. The precipitated esters were dried *in vacuo*, and dissolved in a little HCl. The Ba was removed with Na_2SO_4, and the resulting solution was neutralized to approximately pH 7. These solutions served as substrates for the action of muscle and tumour extracts, respectively. In Table II are given the data of a number of representative experiments of this type. In each case the lactic acid is expressed as mM per 100 ml. substrate solution, after subtracting the appropriate blanks determined for the substrate and enzyme preparations.

The data of Table II show that there is a considerable difference between the fates of the two types of substrate preparation. Muscle extract in every case produced more lactic acid from the muscle substrate preparations than did tumour extract. On the other hand muscle extract failed entirely to form lactic acid from the tumour substrate preparations. As a matter of fact in many instances a negative lactic acid value was found, indicating a disappearance of apparent lactic acid from the muscle extract when it was incubated with the tumour substrate preparation. Tumour extracts were always able to produce

Table II. *Lactic acid formation from NaF substrates* *

Extract no. Muscle	Organic P mg./ml. incubation mixture	Lactic acid produced by	
		Muscle extract m.M/100 ml.	Tumour extract m.M/100 ml.
M 4 A	1·76	0·43	0·10
M 4 B	0·03	0·12	0·07
M 5 A	0·76	0·35	0·25
M 5 B	0·17	0·36	0·31
M 6 A	1·31	0·47	0·01
M 6 B	0·08	0·34	0·10
M 7	0·81	0·69	0·12
		Average 0·39	0·14
Tumour			
T 3 A	1·18	− 0·42	0·18
T 3 B	0·05	0·06	0·30
T 4 A	0·97	− 0·13	0·27
T 4 B	0·11	− 0·10	0·42
T 5	0·76	0·01	0·33
T 6	1·12	− 0·13	0·35
		Average − 0·13	0·31

* I.e. substrates prepared from glycogen by the action of tissue extracts in the presence of NaF.

moderate amounts of lactic acid from these same substrates however. The conclusion seems inescapable that different substances are present in the two substrates, and that different enzymes are involved not only in their formation, but also in the glycolytic reactions.

Nature of the NaF substrates. Superficially the two substrates appear to be composed of much the same type of compound, as is shown by the data of Table III, and as yet we have been unable to isolate the substances responsible

Table III. *Analysis of NaF substrates*

Substrate no.	Organic mg. P/ml.	Phosphate		Reduction mg. glucose/ml.	NaHSO₃ titre ml. 0·005 N
		Hydrolysable			
		30 min.	180 min.		
M 6 A	2·62	8·3 %	23 %	8·6	2·71
6 B	0·15	—	—	2·3	—
T 3 A	1·18	10·6	23·5	5·8	1·64

for the differences in biological behaviour. There is a distinct possibility that some, at least, of these substances may belong outside the group of phosphate esters; because in several instances considerable glycolysis took place in solutions containing small amounts of P (Table II, M5B, T3B, T4B).

The data of Table III give the results of some analyses of three substrate preparations. The reducing values were determined by the procedure of Shaffer & Somogyi [1933]. The NaHSO₃ titre was obtained by adding excess NaHSO₃, oxidizing the excess with I_2, liberating the bound NaHSO₃ with NaHCO₃ and titrating with standard 0·005 N I_2. The rates of hydrolysis of the phosphate esters were studied by estimating the amounts hydrolysed by N HCl after 30 and 180 min. in the boiling water bath. These analyses revealed no obvious differences between the two substrates. The chemical differences indicated by the difference in biological behaviour therefore remain to be demonstrated by fractionation and isolation procedures.

Glycolysis of hexosediphosphate. The glycolysis of hexosediphosphate was studied in some detail by Scharles *et al.* [1935; 1, 2] and by Boyland & Boyland [1935]. After the appearance of these publications, however, a paper was published by Meyerhof & Kiessling [1935–6] which shed new light on the glycolysis of this substance by muscle enzyme systems. In brief, it was found that triosephosphate, formed rapidly from hexosediphosphate in muscle enzyme systems, reacts vigorously with pyruvic acid with the simultaneous formation of phosphoglyceric and lactic acids. The phosphoglyceric acid was found to yield pyruvic acid with the concomitant esterification of glucose to hexosediphosphate. In this way a continuous production of lactic acid could ensue. The reaction involving triosephosphate and pyruvic acid is the key step in this scheme. Indeed, it was the first instance in which the velocity of lactic acid formation from supposed intermediates was found to be as rapid as that from glycogen itself. It was important, therefore, to determine whether tumour extracts are capable of catalysing this reaction.

Formation of triosephosphate. The splitting of hexosediphosphate into two molecules of triosephosphate in muscle extracts [Meyerhof & Lohmann, 1934], was found to occur also in tumour extracts [Boyland & Boyland, 1935]. We have been able to confirm this finding and to show that when tumour extracts are incubated with hexosediphosphate a considerable fraction of the organic P is maintained in the triosephosphate form. The results of one of five such experiments are shown in Table IV. 24 ml. Na hexosediphosphate solution containing 0·881 mg.

Table IV. *Fate of hexosediphosphate during glycolysis by tumour extract*

Phosphate mg. P/ml. filtrate

Time min.	Total	Inorganic	Triose-	Organic	Non-hydrolysable
0	0·731	0·106	0·009	0·625	0·087
1	0·737	0·111	0·059	0·626	0·087
5	0·731	0·117	0·119	0·614	0·086
140	0·743	0·261	0·149	0·476	0·072
200	0·758	0·305	0·150	0·453	0·060
960	0·780	0·653	0·016	0·127	0·046

P/ml. were incubated at 50° with 5 ml. tumour extract for the time indicated. The reaction was then interrupted by the addition of 4 ml. 40 % trichloroacetic acid. The solution was then cooled rapidly and analysed for total, inorganic and easily hydrolysable P, and for that hydrolysed in 180 min. at 100° in N HCl. In several instances the easily hydrolysable P was estimated after preliminary treatment with iodine and sodium carbonate. It was found to be unchanged in each instance, a finding which indicated the absence of glyceraldehyde phosphoric acid.

Effect of pyruvate. The rate of formation of lactic acid from hexosediphosphate was always increased markedly by the addition of pyruvate when muscle enzyme was used, but remained entirely unaffected when tumour enzyme was used. These facts are shown by the data in Table V which represent one of six similar experiments. The solutions were incubated 2 hr. at the temperature indicated. The pyruvic acid was freshly neutralized (with $NaHCO_3$ solution) and added in amounts equivalent, molecule for molecule, to the hexosediphosphate present.

The failure of tumour extracts to catalyse the reaction between triosephosphate and pyruvate indicates, we believe, a significant difference between the glycolytic processes of tumour and muscle systems. Whether or not this difference is due to the absence of the specific coenzyme which is necessary for this

Table V. *Glycolysis of hexosediphosphate in the presence of pyruvate*

Extract	Temp.	Hexose-diphosphate mg. P/ml.	Addition	Lactic acid formed m.M/100 ml.
Rat muscle	40°	0·42	—	0·62
		0·42	—	0·58
		0·42	Pyruvate	0·92
		0·42	Pyruvate	0·97
Mouse sarcoma 180	40°	0·34	—	0·53
		0·34	—	0·54
		0·34	Pyruvate	0·52
		0·34	Pyruvate	0·54
Mouse sarcoma 180	55°	0·44	—	0·87
		0·44	—	0·84
		0·44	Pyruvate	0·84
		0·44	Pyruvate	0·85

reaction in muscle [Meyerhof & Ohlmeyer, 1937] remains to be answered experimentally. That this is true is indicated by the finding of Boyland et al. [1937] that addition of yeast cozymase and adenylic acid to tumour extracts increases their glycolytic activity.

We have found that the rate of lactic acid formation is nearly directly proportional to the concentration of substrate and, within rather wide limits, independent of the amount of tissue extract added (Table VI, representative data from one of three experiments).

Table VI. *Rate of lactic acid formation as affected by the concentrations of enzyme and substrate*

Total vol. 4 ml. Incubated 3 hr. at 40

No.	Sarcoma 180 extract ml.	Hexose-diphosphate mg. P/ml. mixture	Lactic acid formed m.M/100 ml.
1	2	0·44	0·96
2	2	0·22	0·54
3	2	0·11	0·34
4	1	0·44	1·02
5	0·5	0·44	0·91

There is a fundamental difference between the respective extracts used in the two laboratories. Boyland has shown that his aqueous extracts contain coenzyme-destroying enzymes which we find are absent from our saline extracts. This is suggested by the fact that the tumour adenylic acid deaminase can be extracted completely by water, but is absent or nearly absent from saline extracts made in this laboratory [Hitchings & Salter, unpublished]. In short, our experimental data and those of Boyland bear upon different aspects of the general problem.

Mawson [1936] recently has argued that the chief pathway of lactic acid formation by tumour from hexosediphosphate at 52° is via methylglyoxal and the glyoxalase system. This conclusion was based on the increased lactic acid which resulted from addition of glutathione to the extracts at 52°, although this did not occur at 38°. It was based, furthermore, on the fact that addition to the tumour extract of an acetone powder prepared from kidney decreased the yield of lactic acid. The latter observation was interpreted as the result of the anti-glyoxalase activity of the kidney preparation. It is capable however of a simpler

explanation: kidney extracts rapidly hydrolyse hexosediphosphate preparations without producing lactic acid. This is shown by the data of Table VII, which represent one of two similar experiments. A mixture containing 5·96 mg. P as

Table VII. *Action of kidney extracts on hexosediphosphate*

Time min.	Phosphate mg./ml.				Lactic acid mM/100 ml.
	Total	Inorganic	Triose-	Organic	
0	0·650	0·124	0·006	0·526	—
10	0·650	0·330	0·144	0·330	—
21	0·661	0·462	0·082	0·199	0·01
30	0·666	0·534	0·051	0·132	—
45	0·661	0·595	0·025	0·066	– 0·03

hexosediphosphate and 3 ml. 1 : 2 saline extract of mouse kidney tissue was incubated at 55°, total vol. 11 ml. At the stated intervals 2 ml. samples were withdrawn, added to 8 ml. 6 % trichloroacetic acid, cooled immediately in an ice-salt bath, centrifuged and analysed. Parallel experiments were used for the estimation of lactic acid. It is obvious from these experiments that kidney preparations would reduce the lactic acid formation from hexosediphosphate by other tissue extracts, simply because of their phosphatase activity.

DISCUSSION

The finding that tumour extracts fail to catalyse the reaction between triosephosphate and pyruvic acid which plays such an important part in the accepted scheme for muscle glycolysis [Meyerhof & Kiessling, 1935–6] is further confirmation of the opinion of Scharles et al. [1935: 1, 2] that glycolytic reactions in muscle and tumour follow different pathways at certain stages. This conclusion is confirmed also by the finding that different products are formed from glycogen by the two respective tissue extracts in the presence of phosphate and NaF. With respect to the pyruvic acid mechanism we are in agreement with Tsuzuki [1936] who found the mechanism for its formation and utilization (in conjunction with glycerophosphate) to be defective in at least three essential stages.

The glyoxalase mechanism [Mawson, 1936] of tumour glycolysis seems to us to be of doubtful importance as a physiological pathway, both because it is demonstrable only at unphysiological temperatures and also because its occurrence in such a wide variety of tissues [Mawson, 1937] indicates that it has little to do with the peculiarities of tumour glycolysis.

CONCLUSIONS

Differences in the pathways of muscle and tumour glycolysis can be demonstrated:

(1) in the properties of the products formed on incubating glycogen with the respective tissue extracts in the presence of phosphate and NaF, and

(2) in the failure of tumour extracts to catalyse the reaction between triosephosphate and pyruvate.

We wish to express our indebtedness to the Winthrop Chemical Company for a generous gift of candiolin (calcium hexosediphosphate).

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CLXXXVI. ANIMAL PHENOLASES AND ADRENALINE

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RECENT work on the enzymic oxidation of adrenaline has suggested that the animal phenolases might play a physiological role in the inactivation of adrenaline liberated at adrenergic nerve endings [Green & Richter, 1937; Bacq, 1938]. Since data are available as to the distribution of the chromaffin system in the invertebrates it appeared that a comparative study of the distribution of the phenolases would help to show if there is any relation between the two systems.

Heard & Raper [1933] showed that adrenaline is oxidized by the catechol oxidase of mealworms and Neuberg [1908] described a phenolase in *Sepia* which inactivates adrenaline. The literature contains a number of other references to the occurrence or absence of phenolases in particular animal tissues [Oppenheimer, 1926; Pugh, 1934] but quantitative data as to the amount of enzyme present are lacking and the enzymes are often ill-defined so that it is not clear to what extent they would oxidize adrenaline. The older workers generally used the colour formation on incubating in the air with an oxidizable phenol such as catechol or quinol as a method of testing for phenolases, but in many cases the pH was inadequately controlled so that the enzymic nature of the oxidations described appears doubtful. This method is further unreliable since small amounts of reducing substances such as ascorbic acid or glutathione in the tissue preparations may prevent the appearance of colour.

In the present work the manometric method of measuring the oxygen uptake was used. Preliminary experiments were first carried out with tissue preparations obtained from a series of animals of different phyla in order to obtain an approximate value for the amounts of enzymes of the catechol oxidase type which are present. Homocatechol (3:4-dihydroxytoluene) was used for this purpose in preference to catechol as it is more rapidly oxidized by catechol oxidase and is more closely related to adrenaline and the other naturally occurring catechol derivatives, which generally have a carbon chain attached to the catechol nucleus.

Oxidation of homocatechol by animal tissues

The tissue preparations were made by grinding the tissues for 20 min. with sand, adding 2 vol. of $M/15$ phosphate buffer, centrifuging for 5 min. and making the resulting solution up to a known volume with $M/15$ buffer solution. With tissues such as liver which show a rapid spontaneous O_2 uptake the extract was dialysed and 1/10 vol. $M/1.5$ buffer then added before diluting. Buffer of pH 7·4 was used except where otherwise stated. With *Aurelia* the extract was buffered with 1/10 vol. $M/1.5$ buffer but was not otherwise diluted. The O_2 uptakes were measured by the Warburg method; each cup contained 2 ml. tissue extract and

0·2 ml. water or $M/4$ homocatechol solution. The homocatechol was prepared from vanillin by reduction and demethylation and had B.P. 135°/12 mm. With tissues containing active enzymes the extract was diluted 1 : 6 or 1 : 12 and the O_2 uptake during the first 4 min. was measured. With the relatively inactive tissues the extract was used undiluted and the O_2 uptake was measured during 1 hr. The O_2 uptakes are given in Table I as μl. O_2/g. fresh tissue or blood/hr. Individual specimens often showed considerable variation, but the figures given nevertheless show the order of magnitude of the amount of phenolase present.

With the *Cancer* and *Helix* preparations the oxidation rate showed a slight autocatalytic increase: other systems showed a falling off in the oxidation rate after the first few minutes, but the oxidation rates remained in general approximately constant during the time of measurement. Since the plant phenolases have been more fully investigated than the animal enzymes four plant tissues were included for comparison; experiments showing the effect of adding $10^{-3} M$ HCN are also given.

In these preliminary experiments no attempt was made to study the properties of the purified enzymes but rather to observe their activity in the presence of the other cell constituents. The purified catechol oxidases are comparatively labile and are rapidly inactivated by the reaction products formed in the oxidation of catechol derivatives [Richter, 1934], but the inactivation is much less rapid when other tissue proteins are present. Many of the extracts showed a red colour during the oxidation, but in some cases the colour was masked by that of the extracts themselves. The small O_2 uptakes, of the order of 100 μl. $O_2/g./hr.$, found for many of the tissues in the presence of homo-catechol may be ascribed in part to non-enzymic catalysis by traces of heavy metals and in part to the cytochrome oxidase system which catalyses the slow oxidation of catechol derivatives [Green & Richter, 1937; Keilin & Hartree, 1938, 1]. The large O_2 uptakes, of 3000 or more, observed in several of the arthropods must be attributed to the very active catechol oxidases (also described as "tyrosinases" or "polyphenol oxidases") which are known to occur in animals of this phylum.

In the insect larvae the catechol oxidase is present in the blood but is apparently not confined to the blood since it was found in similar or even higher concentration in the skins which were carefully freed from blood by drying on filter paper, washing with water and pressing on filter paper again. The blood was obtained free from contamination by other tissues by holding the insects firmly between the finger and thumb so as to force the blood towards the middle and then making an incision in the skin.

The vertebrate tissues tested were found to be comparatively inactive, especially in view of the fact that the estimations were done at 37° while 25° was used for most of the other animals. Apart from the arthropods relatively high activity was exhibited only by certain molluscs in which O_2 uptakes of 300–500 $\mu l./g./hr.$ were obtained. This observation of high activity in the arthropods and molluscs appeared specially suggestive in view of the recent work of Kubowitz [1937] and of Keilin & Mann [1938] who have shown that certain plant phenolases are copper-protein complexes, for it is precisely in the arthropods and molluscs that the copper-containing haemocyanins occur. The systems responsible for the oxidation of catechol derivatives in these two phyla were therefore selected for study in greater detail: the crab *Cancer pagurus* and the edible snail *Helix pomatia* served as convenient sources of material.

Table I. *Rate of oxidation of homocatechol*

Phylum	Species	Tissue	Temp. °C.	Tissue alone	With substrate	Difference
					μl. O_2/g. tissue/hr.	
Coelenterata	*Actinia equina* L.	Whole animal	26	0	69	69
	,,	Whole animal (+ HCN)	26	9	61	52
	Tealia felina (L.)	Muscle	24	15	70	55
	,,	Muscle (+ HCN)	24	9	25	16
	,,	Mesenterium	26	60	48	− 12
	,,	Mesenterium (+ HCN)	26	39	32	− 7
	Aurelia aurita (L.)	Denser parts	25	0	2	2
Nematoda	*Ascaris lumbricoides* var. *Suis*	Whole animal (*p*H 7·0)	37	51	100	49
Annelida	*Hirudo medicinalis*	Whole animal (*p*H 7·0)	25	4	130	126
	Lumbricus terrestris	Whole animal (*p*H 7·0)	25	0	55	55
Arthropoda	*Odonestis potatoria* (larva)	Blood	25	0	5340	5340
		Skin	25	0	3320	3320
	Lasiocampa quercifolia (larva)	Blood	25	0	623	623
	,, ,,	Skin	25	0	3680	3680
	,, ,,	Viscera	25	64	1065	1001
	Bombyx quercus (larva)	Blood	25	0	3310	3310
	,, ,,	Skin	25	0	8680	8680
	,, ,,	Viscera	25	278	3060	2782
	Eumorpha elpenor (pupa)	Blood	25	0	5130	5130
	,, ,,	Viscera	25	34	3610	3576
	Locusta migratoria (adult: migratory phase)	Viscera	25	494	2440	1946
	Schistocerca gregaria F. (adult: migratory phase)	Viscera	25	192	1105	913
	Dysdercus intermedius Dist. (adult)	Abdomen	25	239	5080	4841
	Periplaneta americana (adult)	Viscera	25	0	141	141
	Cancer pagurus L.	Blood (*p*H 7·0)	30	18	462	444
	,,	Gills (*p*H 7·0)	30	9	256	247
	,,	Hepatopancreas (*p*H 7·0)	30	27	327	300
	,,	Leucocytes (*p*H 7·0)	30	0	4530	4530
	Homarus vulgaris	Blood (*p*H 7·0)	30	7	290	283
Mollusca	*Helix aspersa*	Viscera	25	23	469	446
	Helix pomatia	Blood	30	35	306	271
	,,	Hepatopancreas	30	48	120	72
	Patella vulgata L.	Viscera	24	35	84	49
	,,	Viscera (+ HCN)	24	38	75	39
Echinoderma	*Echinus esculentus* L.	Intestine	26	9	56	47
	,,	Intestine (+ HCN)	26	7	56	49
	,,	Gonads	26	0	0	0
	Asterias rubens L.	Hepatic caecum	30	7	117	110
	,,	Hepatic caecum (+ HCN)	30	5	102	97
Vertebrata	*Rana temporaria*	Liver	37	18	255	237
	,,	Intestine	37	110	107	− 3
	Guinea pig	Liver	37	27	197	170
	,,	Intestine	37	7	54	47
	Pig	Kidney	37	72	107	35
	,,	Liver (*p*H 7·0)	37	0	129	129
	,,	Pancreas (*p*H 7·0)	37	6	79	76
	,,	Heart (*p*H 7·0)	37	6	116	110
	,,	Intestine (*p*H 7·0)	37	10	66	56
	,,	Spleen (*p*H 7·0)	37	24	51	27
	Sheep	Liver (*p*H 7·0)	37	11	156	145
	,,	Pancreas (*p*H 7·0)	37	14	87	73
	,,	Kidney (*p*H 7·0)	37	12	194	183
	,,	Heart (*p*H 7·0)	37	6	121	115
	,,	Intestine (*p*H 7·0)	37	6	74	68
	,,	Spleen (*p*H 7·0)	37	0	162	162
	,,	Brain (*p*H 7·0)	37	7	29	23
	,,	Thyroid (*p*H 7·0)	37	0	111	111
	Ox	Liver (*p*H 7·0)	37	17	114	97
	,,	Intestine (*p*H 7·0)	37	30	126	96
Plants	*Agaricus campestris*	Basidium	25	35	>4500	4465
	Cichorium intybus	Leaf	25	5	>4500	>4495
	Ricinus communis	Seed	25	3	39	36
	Solanum tuberosum	Tuber	25	7	>4500	>4493

Catalytic systems of Cancer pagurus

Haemolymph system. A system that gave rapid colour formation with catechol or homocatechol and O_2 uptakes of 300–800 μl./g./hr. was present in *Cancer* blood or haemolymph. The activity varied considerably from one animal to another. Gills and hepatopancreas were less active than the blood.

	μl./g./hr.		
	Gills	Hepato-pancreas	Blood
Catechol	186	122	795
Homocatechol	153	300	444

Catechol and homocatechol were used for testing the activity of the preparations and the O_2 uptakes were measured at pH 7·0 and 30°: otherwise the conditions were the same as are described above. The activity showed a marked increase on dialysis: two specimens of blood gave with homocatechol (*a*) undialysed, 284, 444, and (*b*) dialysed, 445, 1180 μl./g./hr. This is apparently due to an inhibitor which is removed by dialysis. Similar systems were also found in the blood of *Homarus vulgaris, Helix aspersa* and the squid *Loligo*, and similar figures were obtained with dialysed and undialysed specimens. The system present in *Cancer* blood had the properties of an enzyme in that it was inactivated by boiling, by digestion with trypsin, by drying or by treating with 95 % alcohol.

Purification. An active preparation was obtained from *Cancer* blood as follows. The animals were cooled to 0° by leaving for a few hours in the ice chest. The blood was obtained by puncturing the heart or severing the dorsal artery and was run directly into centrifuge cups cooled to 0° in ice: each animal gave 50–75 ml. blood. The leucocytes were centrifuged off and the clear liquid was dialysed overnight against distilled water at 0°. The precipitate of protein containing carotenoid pigments which separated was inactive and was centrifuged off. The clear solution containing haemocyanin darkened slowly on keeping but remained unchanged in catalytic activity for several weeks when kept at 0°. Attempts to purify the active system further by precipitation with ammonium sulphate, alcohol or acetone gave rise to gelatinous products which could not readily be centrifuged or filtered.

The catalytic system appeared to be closely associated with the haemocyanin fraction of the blood proteins. Attempts were therefore made to purify the system further by the methods used for crystallizing haemocyanins. To the clear solution obtained by dialysis as described above 5 % acetic acid was added drop by drop with stirring until the solution showed a slight permanent turbidity and gave an orange colour with methyl red (pH 4·7). On dialysing again against distilled water at 0° the protein separated out in the form of a colourless crystalline precipitate. The crystals were small but could be seen to be in the form of hexagonal plates when viewed under the microscope.

The crystals slowly turned a pale greenish blue at the surface on exposure to air. They were insoluble in water but dissolved rapidly and completely in dilute salt solutions. A crystalline protein has not previously been obtained from the blood of *Cancer pagurus*. The protein described, while resembling a haemocyanin in its ready solubility in dilute salt solutions, differed from the haemocyanins in being colourless. The aqueous solution showed a very pale greenish colour but did not turn blue or give a measurable O_2 uptake on shaking for 5 min. with air. Copper estimations showed that the protein contained about the right amount of copper for a haemocyanin (Found: Cu, 0·13, 0·12 %). The protein

showed several points of resemblance to the methaemocyanin obtained by Conant *et al.* [1933] by treating haemocyanins with oxidizing agents: it is apparently a modified form of haemocyanin produced during the process of crystallization. The purified crystalline protein showed considerable catalytic activity when tested in the usual way with homocatechol, the activity corresponding to that originally present in the blood: the mother liquor from which the crystals were obtained showed practically no activity. The activity also remained constant on recrystallizing.

Table II. *Catalysis by crystalline copper-protein complex*

	Dialysed blood (33·3 mg./2 ml.)		Crystalline prep. (28·6 mg./2 ml.)	
	µl. O_2/hr.	Q_{O_2}	µl. O_2/hr.	Q_{O_2}
Catechol	415	12·5	466	16·3
Homocatechol	170	5·1	239	8·3
Adrenaline	4	0·1	12	0·4

Although the purified *Cancer* preparation was very active when tested with catechol and homocatechol it was found, surprisingly, that it showed comparatively little activity with adrenaline as substrate. The catalytic activity was strongly inhibited by cyanide and also by the copper reagent cupferron. The inhibitions found at a concentration of 1/2000 inhibitor in the oxidation of catechol under the conditions previously described were: HCN, 95 %; NaF, 43 %; cupferron, 69 %; sodium diethyldithiocarbamate, 9 %; Na_2S, 24 %; 8-hydroxyquinoline, 29 %. The activity of the preparation was not appreciably changed by treating with dilute acetic acid (pH 3·0) for 4 hr. Under these conditions the haemocyanin is known to undergo modification so that the copper, although it is still combined and cannot be removed by dialysis, becomes capable of catalysing the oxidation of thiol derivatives [Pirie, 1931]. In the crystalline preparation the dialysis at pH 4·7 may have already brought about this modification.
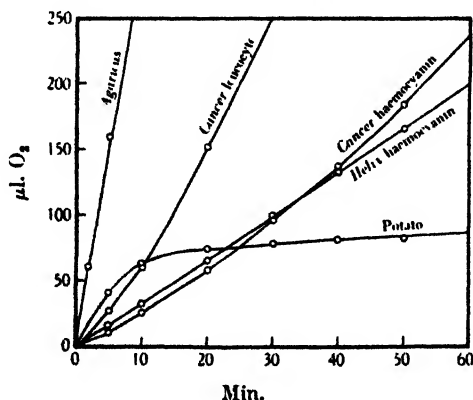
Fig. 1. Oxidation of homocatechol. Conditions as given for Table I. Protein content of preparations in 2·2 ml.: *Agaricus*, 1·7 mg.; *Cancer* leucocyte, 5·9 mg.; *Cancer* haemocyanin, 28·6 mg.; *Helix pomatia* crystalline haemocyanin, 12·0; potato, 0·6 mg.

Cancer leucocyte catechol oxidase. Experiments with crude extracts of gills and hepatopancreas showed that these extracts, unlike the purified preparations from centrifuged blood, catalysed the oxidation of adrenaline. This indicated

the presence of a second catalytic system, which was found in highest concentration in the leucocytes. Pinhey [1930] concluded from colorimetric experiments that crustacean bloods contain a phenolase which is liberated by the bursting of the "explosive corpuscles" of Hardy. In the present investigation Pinhey's observations were confirmed.

The leucocytes were separated from the blood by centrifuging at 0°, washing rapidly with saline and centrifuging again. The extract prepared by grinding with sand in the usual manner contained a very powerful phenolase which catalysed the oxidation of adrenaline as well as that of catechol and homocatechol; the extract also catalysed the oxidation of monohydric phenols such as *p*-cresol.

	μl. O_2/g. tissue/hr.	Q_{O_2} of preparation (μl./mg. dry protein/hr.)
Catechol	2820	49
Homocatechol	4830	83
Adrenaline	5840	100

The Q_{O_2} values obtained for the leucocyte preparations were much higher than with the blood plasma preparations. The enzyme present was similar in activity and specificity to the well-known mealworm catechol oxidase.

Catalytic system of Helix pomatia

In the snail, as in the crab and lobster, the blood contains a system that gives colour formation and O_2 uptake with catechol or homocatechol. Here again the system was closely associated with the haemocyanin fraction of the blood proteins. Crystalline haemocyanin prepared by the method of Dhéré [1919] showed a considerable catalytic activity corresponding to that of the blood. The system was relatively inactive with adrenaline as substrate.

	Dialysed *Helix* blood (14·6 mg. protein in 2 ml.)		Crystalline haemocyanin (12 mg. protein in 2 ml.)	
	μl. O_2/hr.	Q_{O_2}	μl. O_2/hr.	Q_{O_2}
Catechol	160	10·9	134	11·2
Homocatechol	196	13·4	176	14·6
Adrenaline	25	1·7	30	2·5

Catalysis by heavy metals

That traces of heavy metals can catalyse the oxidation of phenols is well known (a good review of the literature is given by Sutter [1936]). In view of their similarity to the plant enzymes the animal catechol oxidases are probably also Cu-protein complexes. The observed catalytic activity of the haemocyanin preparations raises the question of how far the activity of the other phenolases described in the literature may be ascribed to the specific or unspecific catalysis by heavy metals or heavy metal-protein complexes. The activity of the haemocyanin preparations might be attributed to traces of loosely combined Cu present as an impurity in the preparations. It therefore appeared desirable to determine the catalytic activity of traces of heavy metals under the conditions used throughout and to check this against the actual heavy metal content of the preparations.

For several of the substrates tested there was a large increase in the oxidation rate as shown by the colour formation and O_2 uptake on adding Cu, Ni or Co at concentrations of the order of 0·1 mg. metal/ml. Of the commoner heavy metals tested Fe was comparatively inactive as a catalyst; Cu was the most

Table III

O_2 uptakes (μl./hr.) in the presence of heavy metals at pH 7·0 and 30˚. Each Warburg vessel contained 0·2 mg. metal in 2 ml. $M/15$ phosphate buffer and 0·2 ml. $M/4$ substrate. The metals were added in the form of the sulphates.

Substrate	No addition	Cu	Fe	Ni	Co	Mn
Catechol	2	45	12	119	65	13
Homocatechol	34	238	59	313	163	59
Adrenaline	6	115	55	266	251	2
Guaiacol	0	0	0	2	4	0
Protocatechuic acid	2	0	0	8	19	0
Pyrogallol	82	115	42	286	218	76
Phloroglucinol	0	3	0	3	16	3
p-Cresol	0	0	0	0	6	0
Quinol	4	18	2	8	12	20
p-Phenylenediamine	0	198	0	0	135	0
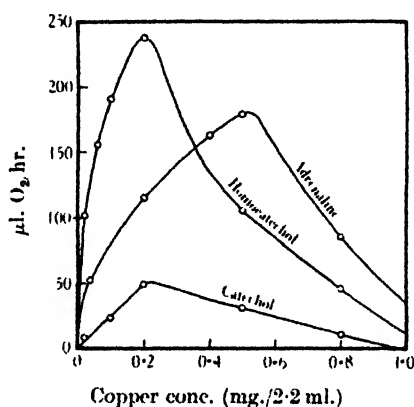
Fig. 2. Effect of Cu concentration on the rate of oxidation. The manometers contained 2 ml. $M/15$ phosphate buffer of pH 7·0 and 0·2 ml. $M/4$ substrate. Experiments at 30˚.

active of those present in significant amounts in most animal tissues. Using Cu as a catalyst the rate of oxidation increased with the Cu concentration (Fig. 2) until high concentrations were reached at which the amount of Cu present was comparable with the amount of phenol, when there was a falling off in oxidation rate. The effect of adding gelatin to the system to form the copper-gelatin complex depended on the substrate and on the Cu concentration, but no very large increase in catalytic activity was observed. The gelatin used was purified by the method of Northrop & Kunitz [1927].

Table IV

O_2 uptakes (μl./hr.) at different Cu concentrations, with and without gelatin. Conditions as in Table III. The figures given for the Cu concentration represent mg. Cu in each Warburg vessel (total vol. 2·2 ml.). The series marked "Gel." contained 7 mg. gelatin.

					Cu concentration						
	0·02		0·05		0·1		0·2		0·5		1·0
Substrate	—	Gel.	—	Gel.	—	Gel.	—	Gel.	—	Gel.	—	Gel.
Homocatechol	102	76	149	100	193	135	238	190	108	177	15	109
Catechol	10	25	14	31	20	38	60	63	34	71	0	38
Adrenaline	46	115	—	—	—	—	115	190	182	225	37	154
Pyrogallol	92	92	—	—	—	—	115	116	133	165	126	220

Although the catalytic activity of Cu is very considerable these experiments show that the activities of the haemocyanin and leucocyte systems are much greater than that of an equivalent amount of inorganic Cu: their activities cannot therefore be due to small amounts of Cu present as an impurity in the preparations. Inorganic Cu is also much less specific in that it catalyses the oxidation of *p*-phenylenediamine, for example, which is not oxidized by the haemocyanin system or the catechol oxidases. This is shown in Table V in which the specificities of a number of phenolases and *pseudo*phenolases are compared.

Table V. *Specificity of catalytic systems*

O_2 uptakes (μl. $O_2/2$ ml./hr.) at pH 7·0 and 30°. Conditions as for Table I.

	Cancer haemo-cyanin	Helix haemo-cyanin	Cancer leuco-cyte	Potato	Agaricus	Dolichos	Cu	Buffer only
mg. protein/2 ml.	27·3	14·6	5·9	0·0	0·3	3·3	0	0
γ-Copper/2 ml.	32·8	32·2	6·5	0·5	0·4	0·3	200	<0·1
Catechol	466	160	286	176	393	229	45	2
Homocatechol	189	196	490	415	385	139	238	34
Adrenaline	21	25	591	210	365	334	115	6
Protocatechuic acid	0	2	3	16	1	5	0	2
Guaiacol	5	0	5	-- -	1	—	0	0
p-Cresol	2	9	742	248	189	0	0	0
Phloroglucinol	2	5	0	72	1	3	3	0
Quinol	12	19	12	36	61	20	18	4
Pyrogallol	231	122	260	185	263	76	115	82
p-Phenylenediamine	12	7	0	9	1	10	198	0

The enzymes were prepared from the mushroom *Agaricus campestris* (basidium), the legume *Dolichos* (seed) and potato tuber by grinding the tissue with sand, extracting with buffer of pH 7·0, precipitating with alcohol and dialysing. The Cu estimations which are included in Table V were done colorimetrically with sodium diethyldithiocarbamate.

The large differences in the specificity of the systems towards different substrates are very striking, and are sufficiently well-defined to be used for identifying them. The *Cancer* leucocyte enzyme shows a specificity similar to those of the mushroom and potato enzymes. Inorganic Cu differs both from the phenolases and from the haemocyanin systems in specificity, but in catalysing the oxidation of *p*-phenylenediamine it resembles the cytochrome system: on other grounds there is evidence that the cytochrome oxidase is a Cu-protein complex [Keilin & Hartree, 1938, 2].

DISCUSSION

(a) *Catalytic activity of haemocyanins.* Catechol and homocatechol gave rapid O_2 uptakes accompanied by colour formation when shaken with the blood of species of *Cancer, Homarus, Loligo* and *Helix*. The natural inference was that this was due to phenolases in the blood, and this appeared to be confirmed when the systems were found to be thermolabile and to show other enzymic properties. Attempts to purify the phenolases then led to the preparation of purified specimens of haemocyanin or Cu-protein complexes derived from the haemocyanins, one of which was obtained crystalline. Crystalline *Helix* haemocyanin also showed considerable phenolase activity. It appeared possible that the catalytic activity might be due to traces of very active phenolase present as an impurity, but this became improbable when it was found that the activity of the haemocyanins remained constant on recrystallization and was higher than that of the unpurified blood proteins. It could be shown that the catalytic activity of the haemocyanins was not due to inorganic Cu contained in the preparations

since an equivalent amount of Cu was much less active and less specific as a catalyst than when combined in the form of haemocyanin. It is therefore concluded that the haemocyanins are themselves catalytically active and can act as *pseudo*phenolases. This *pseudo*phenolase activity of the haemocyanins is comparable with the *pseudo*peroxidase activity of the haemoglobins.

Experiments on the mechanism of the catalysis indicated that a modified form of haemocyanin, possibly methaemocyanin formed during the experiment by the action of traces of *ortho*quinones, is more active than haemocyanin itself since (a) the oxidation curves were autocatalytic in shape, (b) the modified form of haemocyanin produced by treatment with acid was equally active and (c) adrenaline, the *ortho*quinone of which is very labile, was not appreciably oxidized.

The catalytic activity of the haemocyanins is much lower than that of the catechol oxidases, but it is sufficient to give a strongly positive phenolase test by the colorimetric method and to account for the apparent phenolase activities of a number of arthropod and mollusc tissues.

(b) *Distribution of phenolases.* Catechol oxidases are present in a number of arthropods, including the crab *Cancer pagurus* where the enzyme occurs chiefly in the leucocytes. Active phenolases of this type were not found in animals of any other phylum. The number of animals tested is small, but in those hitherto tested apart from the arthropods the results show that if any catechol oxidase is present the amount must be comparatively small.

The literature contains many references to the existence of phenolases in other animal phyla [Oppenheimer, 1926], but the colorimetric technique has generally been used for showing their presence. By the colorimetric method the general practice has been to incubate the tissue extracts at alkaline reaction and 37° with an oxidizable phenol such as "dopa" and examine the extracts after 12–24 hr. for colour formation [Bloch, 1917; Pugh, 1933]. Colour formation under these conditions is an extremely sensitive test which may be given by a number of different types of catalysts. A phenolase which is so inactive that it can be detected only in this way must have quite a different order of activity from the arthropod or plant enzymes, which give high Q_{O_2} values and a coloration with homocatechol at pH 7·0 and 25° in less than a minute.

The present observations on the *pseudo*phenolase activity of Cu-protein complexes show that colour formation in the presence of an oxidizable phenol, even when supported by evidence of thermolability, cannot be taken as sufficient evidence of the existence of a phenolase. Thermolability is unreliable as a criterion of the enzymic nature of a phenolase as small amounts of heavy metals may remain adsorbed on the protein and so may be removed from solution when a protein is coagulated: Stotz *et al.* [1937] showed that artificially prepared Cu-protein complexes resemble enzymes in their thermolability. Cu is present in many animal tissues in sufficient amounts to catalyse the slow oxidation of catechol derivatives; Cunningham [1931] found as much as 0·1–0·9 mg. Cu/g. in a number of animal tissues while 0·025 mg. Cu/g. is sufficient to give a positive colorimetric "phenolase" test with "dopa".

It has recently been shown that the cytochrome oxidase system which is present in many animal tissues can also catalyse the oxidation of catechol derivatives [Green & Richter, 1937; Keilin & Hartree, 1938, 1]. In view of these observations the enzymic nature of a number of the phenolases described in the literature would appear to be doubtful.

(c) *Physiological function of phenolases.* The available evidence gives no support to the view that the catechol oxidases are in any way related to the

chromaffin system in their function. The highest phenolase activity was found in the arthropods, in which no chromaffin system has been found, while *Lumbricus* and *Hirudo* which have well-defined chromaffin systems showed little phenolase activity.

There seems no reason to believe that the *pseudo*phenolase activity of the haemocyanins is of any physiological significance. The very active phenolase of *Cancer* leucocytes, on the other hand, may be expected to come into action by the disintegration of the corpuscles when bleeding owing to an injury occurs [Pinhey, 1930]: the rapid darkening of the shed blood gives evidence of the activity of the liberated phenolase and this is seen particularly clearly with *Maia squinado* and many insects in which the blood rapidly turns nearly black. The physiological function of the phenolases may perhaps be sought in the properties of the *ortho*quinones to which they give rise, such as their activity as respiratory carriers or their bactericidal action.

SUMMARY

1. A number of animal tissues have been examined for the presence of phenolases of the catechol oxidase type.

2. A crystalline Cu-protein complex which is catalytically active has been obtained from the blood of *Cancer pagurus*.

3. Haemocyanins and other Cu-protein complexes can act as *pseudo*phenolases: they are mainly responsible for the apparent phenolase activity in a number of arthropods and molluscs.

4. The catalytic activities of Cu, Fe, Co, Ni and Mn in the oxidation of a series of phenols have been examined.

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CLXXXVII. THE DISTRIBUTION OF THE SUCCINIC OXIDASE SYSTEM IN ANIMAL TISSUES

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IN recent work two functions have been suggested for the enzyme system which catalyses the oxidation of succinate to fumarate in many animal tissues. In muscle and in kidney the oxidation of succinate to fumarate appears to be a step in a cycle of reactions involved in the oxidative breakdown of lactate and pyruvate or of triose [Toenniessen & Brinkman, 1930; Elliott & Schroeder, 1934; Elliott *et al.* 1935; Elliott & Greig, 1937; Krebs, 1937]. According to Szent-Györgyi and his collaborators [e.g. Szent-Györgyi, 1935, 1937] the succinate-fumarate system occupies a central position in the system of oxidation-reduction catalysts which are responsible for tissue respiration, i.e. for the oxidation of cell metabolites in general. The enzymes concerned in succinate oxidation are known to be widely distributed and to occur in high activity in some tissues [Battelli & Stern, 1910]. However, in two types of tumour tissue which respired at a rate comparable with that of normal tissues, Elliott *et al.* [1935] found practically no succinate-oxidizing activity. It therefore seemed that useful information concerning the mechanism of tissue respiration would be obtained by a quantitative survey of the succinate-oxidizing activity and of the enzymic components responsible for succinate oxidation in various types of tissue. The results of such a survey are here presented.

It is generally accepted [Szent-Györgyi, 1924; Fleisch, 1924; Keilin, 1929] that the oxidation of succinate occurs under the influence of two enzymes and a "carrier". One of the enzymes, the dehydrogenase, is specific for succinate and under its influence succinate can be oxidized to fumarate while methylene blue, for instance, accepts H atoms and is reduced to leucomethylene blue. In ordinary circumstances, without methylene blue, the H acceptor is oxidized cytochrome. The cytochrome thus reduced acts as a H carrier and is reoxidized by molecular O_2 under the influence of the long known "indophenol oxidase" which Keilin [1929] has identified with the "respiratory ferment" of Warburg. Following the usual terminology for enzymes, this enzyme is best described as "cytochrome oxidase" since cytochrome is the only known substrate for it. *p*-Phenylene diamine and the "Nadi reagent" (dimethyl-*p*-phenylenediamine + α-naphthol) have been mentioned as substrates for this enzyme, but it seems clear (see below) that these substances also are oxidized through the mediation of cytochrome.

For the purpose of this paper the following terms will be used:

"Succinoxidase" will mean the entire aerobic succinate-oxidizing complex of oxidase, cytochrome and dehydrogenase.

"*p*-Phenylenediamine oxidase" will mean the *p*-phenylenediamine-oxidizing complex of oxidase and cytochrome.

"The oxidase" will mean cytochrome oxidase.

"The dehydrogenase" will mean succinic dehydrogenase. It seems certain that no separate coenzyme is involved in this dehydrogenase activity [Boyland & Boyland, 1934; Andersson, 1934].

Rosenthal [1937] has suggested that the determination of the rate of succinate oxidation in very thin slices of tissue could be used as a means of obtaining a minimum estimate of the respiratory ferment in intact tissue. While the determination of the activity of the succinoxidase in intact cells can best be determined in this way, it will be shown below that the full activity of the respiratory ferment is not displayed unless there is excess of the dehydrogenase and cytochrome present in the cells. Estimates of the full respiratory ferment activity of various tissues have been made by determination of the rate of p-phenylenediamine oxidation by broken up tissues in the presence of excess cytochrome.

EXPERIMENTAL

General methods

Except in a few specified experiments the tissues studied were prepared by "homogenization" of known weights of tissue in definite volumes of $M/10$ phosphate buffer, pH 7·4. The apparatus recently described by Potter & Elvehjem [1936], which reduces tissues rapidly and conveniently to a fine suspension which can be pipetted, was used. In most cases the tissue was chopped up with scissors whilst muscle was passed through a Latapie mincer, before homogenization. With some rather tough tissues the homogenization took several minutes and the homogenizer tube was kept cool in ice water. It was sometimes difficult to break up the last few pieces of tissue without continuing the homogenization for a long time; the weight of these pieces was determined and deducted from the total amount taken. Tissues from freshly killed animals were used; the suspension was kept in a refrigerator between experiments and it was used only during the day on which it was prepared. The suspensions contained amounts of moist tissue ranging from 50 mg. (heart, liver, kidney) to 200 mg. (spleen, tumours) per ml. With many tissues it was necessary to take material from a number of rats to obtain enough suspension for a set of experiments. In every experiment samples of the chopped tissue before homogenizing were weighed and dried at 100–110°, to determine the wet weight/dry weight ratio.

For the determination of O_2 uptake rates, simple Barcroft differential manometers were used [see Dixon, 1934]. The vessels contained air and a total of 3 ml. fluid, of which 1 ml. was $M/10$ phosphate buffer, pH 7·4, added separately or with tissue suspension. Homogenized tissue suspensions were introduced by pipette; in experiments with minced or chopped tissue this was weighed in the dry vessel before adding the solutions. The taps were closed 6 min. after immersing the vessels in the bath at 38° and readings were taken every 5 min. for 25 min. The rate of shaking was 132 oscillations/min. and it was found that O_2 uptakes of well over 1000 μl./hr. could be measured without uncertainty due to diffusion effects. In general, amounts (0·25–2 ml.) of tissue suspension were chosen such that the O_2 uptake was well below 1000 μl./hr. In the large majority of experiments the O_2 uptake/time curve was linear, at least after the first 5 min. The rate was taken from the linear part of the curve or in the few cases where the rate fell off appreciably the initial rate was accepted.

Succinoxidase

For the estimation of succinoxidase activity, equal amounts of the suspension were pipetted into both vessels of the Barcroft manometer and neutralized succinic acid solution was measured into the right-hand vessel. No alkali papers were put in the inner cups since no CO_2 is evolved in the oxidation of succinate to fumarate. Elliott & Schroeder [1934] showed that kidney slices oxidized fumarate rapidly, while with finely minced kidney practically no oxidation with fumarate took place though the succinic oxidation still occurred rapidly. Experiments with homogenized kidney, muscle, and other tissues, showed that no appreciable oxidation of fumarate occurred with the amounts of tissue used and at the concentration of fumarate which was likely to be present.[1]

In order to obtain a comparison of the succinoxidase activities of various tissues, it was first necessary to determine the conditions under which maximum activity would be observed in any tissue. While disintegration of tissue does not destroy succinoxidase, it is known [e.g. Lehmann, 1929] that violent shaking does inactivate it. With tissues having high activity, experiments showed that the activity observed after homogenization by Elvehjem's apparatus was greater than with minced or chopped tissue, probably owing to better dispersion. With several tissues having low activity, homogenizing did decrease the activity somewhat; it is possible that the violent breaking up of these tissues which were all rather tough has some destructive effect on the enzymes appreciable in comparison with the small activity of these tissues (see Table I).

Potter & Elvehjem [1937] carried out some measurements of succinoxidase activity, but the results given by them are considerably lower than those reported below (e.g. for rat kidney and liver suspension they found O_2 uptakes respectively of 5932 and 3895 $\mu l./$hr. per 1000 mg. tissue, against our figures of 22,600–33,200 and 13,300–24,000). Possibly their method of using only 1 ml. of fluid instead of 3 ml., in order to facilitate diffusion, was not quite satisfactory with the high concentration of tissue (40 mg.) which they used. They found that the maximum O_2 uptake occurred with 0·3 % succinic acid in the medium. In this work with all the tissues studied the optimum concentration was found to be about 0·9 %, and this concentration was in general used, though in most cases the O_2 uptake was measured at two or more different concentrations. The rate was often appreciably smaller with 0·6 % or less, or with 1·2 % succinic acid.

It is known that breaking up a tissue into a suspension lowers the respiration considerably compared with the respiration of thin slices. Elliott & Schroeder [1934] suggested that this is due to a dilution of coenzymes etc. and Krebs [1935] and Potter & Elvehjem [1936] showed that with a suspension the rate of respiration per unit weight increases with increasing concentration of tissue.[2] However, Potter and Elvehjem [1936] found that, unlike the residual respiration, the succinoxidase activity of tissues per unit weight is independent of the concentration of the tissue. We have confirmed this with all the tissues studied, by varying the amount of tissue in the 3 ml. of medium; also, in two experiments, the same rate of O_2 uptake was observed with equal small amounts of liver

[1] In later experiments it was found that fumarate increased the O_2 uptakes when higher concentrations of tissue were used. The extra O_2 uptake was largely balanced by extra CO_2 evolution.

[2] With the very dilute suspensions in phosphate buffer solution used in this work, the O_2 uptake in the absence of added substrate varied between 0 and 20 $\mu l.$ in the 25 min. experimental period.

tissue in 3 ml. or in only 1 ml. of medium. Nevertheless, the succinoxidase activity was determined with two or more concentrations of tissue in nearly every case.

It was found that the activity of a liver, kidney, or heart tissue suspension sometimes, but not always, increased up to 35 % (70 % in one instance) during 1 or 2 hr. standing in the refrigerator. After this period the activity remained constant for a long time. Presumably slow solution of constituents of the tissue results in the better dispersion of some constituent of the enzyme system. In nearly every case the succinoxidase activity was determined at least twice,

Table 1. Succinoxidase activities of rat tissues

| | | Succ Q_{O_2} | |
| | | On moist wt. | Mean of homogenized On dry wt. |
Tissue	Preparation		
Whole kidney	Homogenized	33·2, 30·4, 22·6, 18·9	112
Liver	Latapie minced	13·9)	---
	Homogenized	20·2) 24·0, 13·3, 25·8, 21·8, 16·1, 13·8	66
Heart	Chopped	7·3)	---
	Homogenized	18·4) 12·8, 12·9, 13·0, 12·1, 13·2	62
Whole brain	Ground to paste	3·9)	...
	Homogenized	3·8) 4·2, 3·7, 3·2, 3·5, 4·0, 4·6, 4·3, 6·2	18
Testis	Ground to paste	1·6)	---
	Homogenized	1·9) 1·5, 1·2, 1·7, 1·1, 1·9, 2·0, 1·7	13
Adrenal	Homogenized	3·6	11·7
Lung*	Chopped	0·6)	--
	Homogenized	1·8) 1·8, 0·9	7·5
Skeletal muscle	Latapie minced	2·5) 3·1) 2·4)	--
	Chopped	2·3)	--
	Homogenized	1·5) 1·7) 1·4) 1·9, 1·3	6·6
Thymus	Chopped	0·51	---
	Homogenized	0·33	1·6
Pancreas	Chopped	0·24) 0·13)	---
	Homogenized	0·0) 0·02)	0
Spleen	Chopped	0·13)	---
	Homogenized	0·16) 0·23, 0·04, 0·09	0·5
Blood†	Laked	0, 0	0
Whole foetus (1·1 g. each)	Chopped	0·73)	---
	Homogenized	1·90)	17·4
Jensen sarcoma (intramuscular)	Chopped	0·6‡)	---
	Homogenized	2·1) 2·7, 2·1	13
Jensen sarcoma (subcutaneous)	Homogenized	2·0	12·9
Flexner Jobling carcinoma	Homogenized	2·0, 0·4	12·8, 2·1
Walker No. 256 carcinoma	Chopped	0·08)	---
	Homogenized	0) 0·1, 0·1, 0·16, 1·0	0·6, 7·2
Philadelphia No. 1 sarcoma	Chopped	0·13)	---
	Homogenized	0·11) 0·40, 0	1·0
Spontaneous mammary carcinomata	Chopped	0·21)	---
	Homogenized	0·03) 0·18	0·5

* The O_2 uptake rates of rat and rabbit lungs with succinate fell off rapidly with time.
† The blood was taken from the hearts of etherized animals and run into water containing a trace of heparin. Tests showed that neither traces of ether nor of heparin affected succinoxidase.
‡ Tissue clumped together and rate fell off rapidly.

Table II. *Succinoxidase activities of rabbit and other tissues*

Tissue	Preparation	Succ Q_{O_2} On dry wt.	
Rabbit kidney cortex	Homogenized	65·7	
Rabbit kidney medulla	Homogenized	24·5	
Rabbit liver	Latapie minced	34·5	
	Homogenized	36·2	
Rabbit heart	Homogenized	150	
Rabbit testis	Homogenized	9·2	
Rabbit lung	Chopped	6·1	
	Homogenized	8·3	
Rabbit skeletal muscle	Latapie minced	2·1	
	Homogenized	1·5	
Chick embryo (7 day)	Chopped	0·7	
	Homogenized	2·0	
Ox retina	Intact	10·3	3·9
	Homogenized	10·1	11·9

at intervals of 1 or 2 hr. When this was not convenient the determination was made 2 or 3 hr. after preparing the suspension.

To express succinoxidase activity the term Succ Q_{O_2} will be used where

$$\text{Succ } Q_{O_2} = \frac{\mu\text{l. } O_2 \text{ taken up in the oxidation of succinate}}{\text{hr. } \times \text{ mg. tissue}}.$$

Tables I and II give the activities found in a number of tissues. The results are calculated on the moist weight and on the dry weight of tissue taken, the dry weight being deduced from the wet weight/dry weight ratio of the separately dried sample of tissue.

It will be seen in Tables I and II that the distribution of activity is very similar in the tissues of the rabbit and the rat. According to their succinoxidase activities the tissues fall roughly into three groups. (1) Kidney, liver and heart muscle have very high activities.[1] (2) Brain, testis, skeletal muscle, lung, adrenal, retina, Jensen sarcoma and Flexner-Jobling carcinoma, have fairly low activities. (3) Thymus, pancreas, spleen, blood, Philadelphia No. 1 sarcoma (usually), Walker No. 256 carcinoma, and two spontaneous mammary carcinomata (rat) have only a trace of activity or none at all. Battelli & Stern [1910] noted the high activities of kidney, liver and heart, but the activities observed with the crude methods available to them were several times lower than those now found. At present there does not seem to be any clear connexion between metabolic activity and succinoxidase activity. It is interesting to find tissues with normal respiratory activity but with practically no succinoxidase, as with spleen and several tumours. Absence of succinoxidase is not a definite characteristic of cancer tissue since some types of tumours, e.g. the Jensen sarcoma, always have definite activity. Fleisch [1924] and Boyland & Boyland [1936] have also found succinoxidase in the Jensen sarcoma. It does not seem to be a characteristic even of a single strain since with the Walker No. 256 one crop[2] was found to be quite active.

[1] The activity of these tissues is so high that very dilute suspensions of them are useful as quickly obtainable preparations of "succinoxidase" for use in rough estimations of succinate.

[2] For a set of experiments with small tumours, five or six young tumours implanted at the same time in several rats from one tumour were used.

The dehydrogenase

The succinoxidase activity as measured in the previous section is liable to be limited by the amount of any one of the components of the catalytic system. It was of interest, therefore, to obtain separate estimates of the specific succinate enzyme, i.e. the dehydrogenase, by an adaptation of the well-known methylene blue reduction technique of Thunberg. In the reduction of methylene blue no O_2 activator or carrier is concerned and only the rate of H transfer from succinate to methylene blue under the influence of the dehydrogenase is measured.

The Thunberg tubes used were of the Keilin [1929] type with a bulb in the stopper. The stopper bulbs contained 0·5 ml. 0·703 % methylene blue, this concentration being chosen so that 0·475 ml. contains methylene blue equivalent as H acceptor to 10 μl. O_2. The main tube contained a total of 2·5 ml. fluid consisting of tissue suspension in $M/10$ buffer, neutral succinate solution, water and enough extra buffer to make with the tissue suspension a total of 1 ml. phosphate buffer ($M/10$, pH 7·4). Control tubes contained the same additions with water instead of succinate solutions. Up to 10 tubes were evacuated simultaneously by means of a brass tube with 10 side tubes, connected to an oil suction pump and manometer. The tubes were evacuated three times and refilled with N_2 freed from O_2 by passage through a tube of hot copper wire fragments. They were finally evacuated, firmly closed, and the side tubes filled with water. In order to prevent the tissue from settling out, the tubes were fixed into a series of clips on a horizontal beam which was attached to the shaking apparatus of an ordinary Barcroft manometer tank; the tubes used were bent through an angle of about 40° and the clips were fixed at such an angle that the main parts of the tubes were nearly horizontal and the motion of the shaker kept the contents of the tubes well mixed. The main parts of the tubes were immersed in the bath water at 38°. The tubes were shaken in the bath for 1–3 min. before mixing the methylene blue at zero time. 1 min. was found to be sufficient to bring the fluid almost to the temperature of the bath. In open test tubes, mixtures were made of the same amounts of tissue suspension and buffer as in the Thunberg tubes, with water added to make a total of 3 ml., and 0·025 ml. of the methylene blue solution were added. These comparison tubes were frequently shaken with air to keep the methylene blue oxidized. The time of reduction was taken as the time when the colour in the Thunberg tube matched the colour in the comparison tube containing the same amount of tissue.

The activity of the succinic dehydrogenase will be expressed by the term Succ Q_{MB} where

$$\text{Succ } Q_{MB} = \frac{\mu\text{l. } O_2 \text{ equivalent of methylene blue reduced by the succinate system}}{\text{hr.} \times \text{mg. tissue}}$$

$$= M \frac{t - t_{Succ}}{t} \times \frac{60}{t_{Succ} \times w},$$

where $M = O_2$ equivalent of the total methylene blue reduced,

t = time in min. for reduction in the absence of succinate,

t_{Succ} = time in min. for reduction in the presence of succinate.

$\frac{t - t_{Succ}}{t}$ = fraction of total methylene blue reduced which is reduced by succinate,

w = weight of tissue in mg.

Table III. *Examples of the determination of succinic dehydrogenase activity of homogenized rat tissues*

Tissue	mg. moist	t	t_{Succ}	Succ Q_{MB}	Tissue	mg. moist	t	t_{Succ}	Succ Q_{MB}
Kidney	20	∞	8, 8, 8	3·8	Muscle	200	23, 28	8·5, 8·5, 8·5	0·24*
	10	∞	15, 15	4·0		100	77, 84	14·5, 14·5, 14·5	0·34
	5	∞	38, 38, 43	3·0		50	200 ±	24, 21·5, 22	0·47
		6 hr. later					4 hr. later		
	20	∞	8, 8, 8	3·8		100	72, 75	14, 14·5	0·34
	10	∞	13, 16, 16	4·0		50	200 ±	23, 23	0·46
	5	∞	32, 34, 34	3·7		25	500 ±	51, 51, 49	0·46
Liver	50	109	6, 5·5	2·0	Spleen	200	10·5, 10·5	7, 7	0·14*
	25	360 ±	12, 12	1·9		100	32, 32	12, 12	0·31
	12·5	∞	23, 26	2·0		50	155 ±	29, 28	0·34
		3 hr. later			Blood	240	∞	∞	0
	12·5	∞	27, 27	1·8		120	∞	∞	0
Heart	50	210 ±	3·5, 3·5	3·4*		60	∞	∞	0
	25	350 ±	6, 6	3·9	Jensen sarcoma	133	50, 40	15, 15, 16	0·20
	12·5	∞	10·5, 10·5	4·6		70	120, 120	38, 31	0·18
	12·5	∞	12, 11·5	4·1		35	∞	105, 89	0·12†
	6·25	∞	25, 26	3·8	Walker No. 256 carcinoma	195	40, 48	19·5, 14·5	0·11
	3·13	∞	67, 67	2·9†		98	190 ±	49, 59	0·08
Brain	100	35, 40	5·5, 5·5	0·95	Phila. No. 1 sarcoma	200	7·5, 6·5	5·5, 5, 5·5	0·15*
	50	126, 141	12·5, 13	0·86		160	25, 29	8, 8	0·33
	25	600 ±	26, 26	0·88		100	86, 74	22·5, 22·5	0·19
Testis	200	23, 21·5	8·5, 8·5	0·22		80	108 ±	46, 46	0·09†
	100	60, 61	20, 19	0·20					
	50	425 ±	43, 39	0·26					
Lung	200	27, 35	7, 7	0·37					
	100	171 ±	34, 34	0·16†					
	50	∞	83, 83	0·16†					

Accurate determination of the reduction time with spleen is difficult due to the large amounts of haemoglobin present which is reduced in the vacuum tubes and oxygenated in the comparison tubes.

* The method often does not show the maximum activity when the reduction time is very short.

† With long reduction times inactivation of the enzyme seems to occur. With lung this is particularly marked. The succinoxidase activity of lung (rat and rabbit) also falls off rapidly in aerobic experiments.

Table IV. *Succinic dehydrogenase activities of homogenized rat tissues*

Tissue	Succ Q_{MB}* On moist wt.	Mean on dry wt.	Tissue	Succ Q_{MB}* On moist wt.	Mean on dry wt.
Kidney	3·9, 2·2	12·9	Muscle	0·42, 0·51, 0·59	2·1
Liver	2·0, 1·3, 1·8, 1·4	5·1	Spleen	0·25, 0·31, 0·32	1·3
Heart	3·4, 4·1	17·1	Blood	0	0
Brain	0·81, 0·88	3·9	Jensen sarcoma	0·20, 0·22	1·3
Testis	0·16, 0·23	1·5	Walker No. 256 carcinoma	0·06, 0·10	0·5
Lung†	0·32, 0·38	1·8	Phila. No. 1 sarcoma	0·22	1·3

* Widely variant results, see footnotes to Table VII, omitted.

† Uncertain, see footnote to Table III.

Under the conditions chosen for these experiments, $M = 10$,

whence $$\text{Succ } Q_{\text{MB}} = \frac{600}{t_{\text{Succ}} \times w} \times \frac{t - t_{\text{Succ}}}{t},$$

or when the reduction by the tissue alone is very slow compared with the time in the presence of succinate, i.e. when t is much greater than t_{Succ},

$$\text{Succ } Q_{\text{MB}} = \frac{600}{t_{\text{Succ}} \times w}.$$

This formula is of course only an approximation, since it is assumed that the rate of reduction by substrates in the suspension is unchanged by the presence of succinate, and that the rate of reduction by succinate and other substrates is independent of the methylene blue concentration. Further uncertainty may be introduced by adsorption of the dye on tissue particles, and by progressive inactivation of both the succinic dehydrogenase and the other reducing mechanisms. However, the method serves to give a rough comparison of the dehydrogenase activities of the various tissues. While the time of reduction without succinate (t) tends to increase very rapidly with dilution of the tissue, the succinic dehydrogenase activity as measured by this method is usually approximately proportional to the amount of tissue. Occasionally rather wide variations in Succ Q_{MB} were found, especially with tissues of low activity, or when the reduction time was very short or very long. No appreciable change in activity was noticed in suspensions kept in the ice box for a few hours. Table III illustrates these points. The optimal concentration of succinate was found to be about 0·2–0·5 %; with very low concentrations the maximum rate is not reached, and higher concentrations cause definite inhibition. It will be noticed that the optimal concentration of succinate is lower for the reduction of methylene blue than for O_2. It is possible that excess succinate causes over-saturation of the enzyme surface preventing access of the dye, while with O_2 the reaction with the carrier and oxidase is so much more rapid that succinate is removed from the dehydrogenase surface rapidly enough to prevent oversaturation at this concentration.

For routine determinations, 10 mg. succinic acid in the 3 ml. (0·33 %) were used. Tubes were set up in duplicate or triplicate and two or more different amounts of tissue were used; usually amounts which would give reduction times 8–35 min. in the presence of succinate were chosen. In Table III examples of dehydrogenase determinations are shown and the results of determinations on a number of tissues are summarized in Table IV.

The value for Succ Q_{MB} is always much lower than Succ Q_{O_2} [see also Bach & Michlin, 1927; Wieland & Frage, 1929], which indicates that the reaction occurs less readily with methylene blue than with the natural carrier, cytochrome. The various tissues fall roughly into the same order according to dehydrogenase activities as they do according to their complete succinoxidase activities. However there is no constant relation between Succ Q_{MB} and Succ Q_{O_2}, a point which will be discussed further in a later section.

p-*Phenylenediamine oxidase*

Battelli & Stern [1912, 1] showed that all the tissues of higher animals have the power of oxidizing p-phenylenediamine and showed the variation of activity between different tissues. Several authors [Holmes, 1930; Penrose & Quastel, 1931; Quastel & Wheatley, 1932] have used the rate of oxidation of p-phenylenediamine as a measure of the "indophenol oxidase" or "respiratory ferment"

activities of various tissues. Quastel and his collaborators showed that the activity of the oxidase is not affected by destruction of the cell structure. In the next section it will be shown that since both the oxidase and cytochrome are concerned in the oxidation of p-phenylenediamine a true estimate of the full oxidase activity of tissues is not obtained unless excess cytochrome is present. However, the p-phenylenediamine oxidase, i.e. the oxidase plus cytochrome as present in the tissue suspensions, has been estimated in a number of tissues.

The procedure was similar to that for measuring succinoxidase activity. The p-phenylenediamine solution, freshly prepared each day, was added to both vessels of the manometer to compensate for the small autoxidation of the base (20–30 μl. per hr.). Tissue suspension was added only to the right-hand vessel. In a separate experiment the residual respiration of the tissue suspension at the concentration used was determined. This was always negligibly small and it was not deducted from the O_2 uptake observed with p-phenylenediamine since most of it probably takes place, like the p-phenylenediamine oxidation, through the mediation of the oxidase and cytochrome.

The rate of O_2 uptake increases rapidly with increasing p-phenylenediamine concentration up to about 0·6 %, and the optimum concentration was found to be about 1 %, further increase often producing a slight inhibition. In all estimations, therefore, the vessel fluid contained 30 mg. of the base in the 3 ml. In some cases the rate of p-phenylenediamine oxidation per unit weight of tissue increased somewhat with increasing concentration of tissue. Examples of this effect are shown in Table V. Duplicate determinations of O_2 uptake rates with p-phenylenediamine within a few hours often varied somewhat, but no constant effect of time of standing suspensions was noticed.

To express the rate of p-phenylenediamine oxidation, the term p-Phen Q_{O_2} will be used, where

$$p\text{-Phen } Q_{O_2} = \frac{\mu l.\ O_2\ \text{taken up in the oxidation of } p\text{-phenylenediamine}}{\text{hr.} \cdot \text{mg. tissue}}.$$

In Table VI the results obtained with various tissues are summarized. Again the order of the activity of the various tissues is about the same as for succinoxidase and the dehydrogenase. The activities found are several times higher than those given by Battelli & Stern [1912, 1] to whom only crude methods were available and who used tissues kept 5 hr. before mincing. As was noticed by Battelli & Stern, the rate of succinate oxidation by liver and kidney is somewhat higher than that of p-phenylenediamine (see Table VII). Both p-phenylenediamine and the succinate-dehydrogenase system react readily with cytochrome c, but it is possible that, with some tissues, structural arrangements on the tissue particles enable the latter system to react more rapidly than p-phenylenediamine with the insoluble cytochromes, a and b.

It is probable that both p-phenylenediamine and succinate-dehydrogenase can react with cytochromes a and b since in experiments in which the amount of soluble cytochrome was reduced by repeated washing of the tissue (brain, testis) the relative fall in O_2 uptake was small and about the same with either substrate.

The case of blood is exceptional. Whole rat blood diluted (and cytolysed) with water was used. With constant p-phenylenediamine concentration (30 mg. in 3 ml.), the O_2 uptake rate increased with increasing blood concentration up to about 200 mg. blood/3 ml.; further increase in blood concentration caused no further increase in O_2 uptake rate. The rate was steady, and it was quite low so that O_2 diffusion could not have been the limiting factor—increasing the surface by speeding up the shaking had no effect. It was noticed that the absorption

Table V. *Effects of tissue concentration and added cytochrome concentration on the p-phenylenediamine oxidation of rat tissues*

p-Phen Q_{O_2} (on moist wt.)

Tissue	Cytochrome added mg.	mg. tissue in 3 ml															
		3.1	5	6.3	10	12.5	20	25	37.5	40	50	75	100	150	200	300	400
Kidney	—	—	—	—	—	24.6	—	24.9	—	—	27.9	—	—	—	—	—	—
	1.6	—	—	—	—	68.5	—	68.8	—	—	—	—	—	—	—	—	—
	—	—	—	—	—	—	21.8	—	—	23.7	—	—	—	—	—	—	—
	1.6	—	74.4	—	64.6	—	54.2	—	—	—	—	—	—	—	—	—	—
	3.2	—	72.0	—	66.7	—	57.8	—	—	—	—	—	—	—	—	—	—
Liver	—	—	—	—	—	—	—	12.5	—	—	12.8	11.9	—	—	—	—	—
	—	—	—	—	—	—	—	—	10.9	—	—	10.8	—	—	—	—	—
	1.6	—	—	—	50.5	—	46.1	—	38.0	—	—	—	—	—	—	—	—
	3.2	—	—	—	51.2	—	46.8	—	40.6	—	—	—	—	—	—	—	—
Heart	—	—	—	—	—	15.4	—	16.4	—	19.1	—	—	—	—	—	—	—
	—	—	—	—	—	23.2	—	25.6	—	—	—	—	—	—	—	—	—
	1.6	—	—	112.0	—	95.0	—	—	—	—	—	—	—	—	—	—	—
	—	—	—	—	—	25.0	—	27.5	—	—	28.8	—	—	—	—	—	—
	1.5	97.8	—	94.9	—	91.8	—	—	—	—	—	—	—	—	—	—	—
	3.0	114.5	—	116.5	—	112.6	—	—	—	—	—	—	—	—	—	—	—
Brain	—	—	—	—	—	—	—	8.8	—	—	9.3	11.3	12.4	—	—	—	—
	0.7	—	—	—	—	—	—	15.0	—	—	14.6	—	—	—	—	—	—
	1.4	—	—	—	—	—	—	15.1	—	—	15.3	—	—	—	—	—	—
	—	—	—	—	—	—	—	—	6.9	—	—	6.8	—	—	—	—	—
	1.5	—	—	—	29.5	—	20.7	—	24.1	—	—	—	—	—	—	—	—
	3.0	—	—	—	29.5	—	28.7	—	25.2	—	—	—	—	—	—	—	—
Testis	—	—	—	—	—	—	—	—	—	—	—	—	2.9	—	3.2	—	—
	0.7	—	—	—	—	—	—	—	—	—	—	—	6.9	—	6.5	—	—
	1.4	—	—	—	—	—	—	—	—	—	—	—	8.3	—	—	—	—
	—	—	—	—	—	—	—	—	—	—	—	—	—	3.2	—	2.8	—
	1.5	—	—	—	—	—	—	—	12.7	—	—	11.1	—	—	9.2	—	—
	3.0	—	—	—	—	—	—	—	13.1	—	—	11.5	—	10.0	—	—	—
Lung	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1.1	—	1.0
	1.0	—	—	—	—	—	—	—	—	—	—	—	—	—	5.7	—	—
	2.0	—	—	—	—	—	—	—	—	—	—	—	—	—	6.8	—	—
Skeletal muscle	—	—	—	—	—	—	—	—	—	—	—	2.8	—	—	3.5	—	—
	0.5	—	—	—	—	—	—	—	—	—	—	9.3	—	—	9.3	—	—
	1.0	—	—	—	—	—	—	—	—	—	—	10.1	—	—	10.1	—	—
Spleen	—	—	—	—	—	—	—	—	—	—	—	—	1.6	—	1.4	—	—
	0.7	—	—	—	—	—	—	—	—	—	—	—	5.6	—	—	—	—
	1.4	—	—	—	—	—	—	—	—	—	—	—	6.7	—	—	—	—
Jensen sarcoma	—	—	—	—	—	—	—	—	—	—	—	—	3.3	—	3.9	—	—
	—	—	—	—	—	—	—	—	—	—	—	—	1.8	—	1.6	—	—
	1.5	—	—	—	—	—	—	8.1	—	—	—	6.6	—	5.2	—	—	—
	3.0	—	—	—	—	—	—	8.0	—	—	—	7.4	—	5.8	—	—	—
Phila. 1 sarcoma	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.8	—	0.8
	1.0	—	—	—	—	—	—	—	—	—	—	—	—	—	4.6	—	—
Blood	—	—	—	—	—	—	—	6.6	—	—	—	4.6	2.8	—	1.5	—	0.7
	1.0	—	—	—	—	—	—	6.9	—	—	—	—	—	—	1.5	—	—

Table VI. p-*Phenylenediamine oxidase activities of rat tissue suspensions*

Tissue	p-Phen Q_{O_2} On moist wt.	Mean on dry wt.	Tissue	p-Phen Q_{O_2} On moist wt.	Mean on dry wt.
Kidney	16.4, 17.7, 21.9, 20.0, 24.7, 23.7	91	Skeletal muscle	2.9, 1.8	10
			Spleen	1.5, 1.3	6.2
Liver	11.9, 10.9, 10.4, 13.6, 14.1, 10.2, 11.9, 10.9	40	Pancreas	0.5, 0.3	1.0
Heart	20.5, 19.1, 23.4, 16.4, 18.8, 23.5, 27.5	96	Jensen sarcoma	2.2, 2.9, 3.3, 1.3, 1.7	14
			Flexner-Jobling	1.1, 0.6	5.2
Brain	8.4, 8.1, 8.9, 10.7, 8.4, 9.8, 9.8, 9.3, 6.8	43	Walker No. 256	0.5, 0.3, 0.5, 0.4,	2.6
Testis	3.0, 2.8, 3.1, 3.5, 3.2, 3.4, 2.5, 2.5, 3.1	24	Phila. No. 1	0.8, 0.7	4.2
Lung	1.1, 1.2	5.8	Blood (maximum observed)	6.6	124
Retina (Ox)	1.5	13			

bands of oxyhaemoglobin could be observed with the higher amounts of blood, they were faint at the limiting concentration, and completely absent in the range where O_2 uptake was proportional to blood concentration. In the latter solutions it appeared that the haemoglobin was completely destroyed: no methaemoglobin band in the red could be seen nor was reduced haemoglobin regenerated on the addition of hydrosulphite. Addition of cytochrome had no effect on the oxidation. When the concentration of blood was kept constant at a high value (450 mg./3 ml.), the O_2 uptake increased greatly with increasing p-phenylenediamine concentration tending to a maximum rate at a very high concentration of the base. It seems probable that the actual catalyst of the oxidation is a breakdown product of haemoglobin formed by a reaction between haemoglobin and p-phenylenediamine, and the amount formed is a function of both haemoglobin and p-phenylenediamine concentrations. Entirely similar results were obtained with crystalline horse haemoglobin: these results are shown in Figs. 1 and 2. The haemoglobin solutions were estimated by the ferricyanide method.
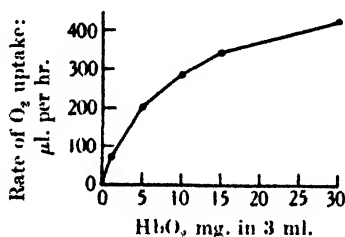
Fig. 1.
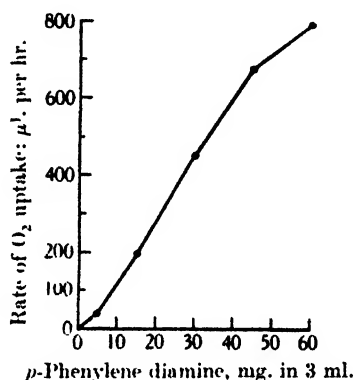
Fig. 2.

Fig. 1. Effect of varying HbO_2 concentration, with constant (30 mg.) p-phenylenediamine.

Fig. 2. Effect of varying p-phenylenediamine concentration, with constant (30 mg.) HbO_2.

Cytochrome

No attempt has been made to estimate cytochrome in the tissues directly, but some information about the quantitative relations of oxidase, cytochrome and dehydrogenase in tissue suspensions has been obtained by experiments in which known amounts of cytochrome were added.

Solutions of pure oxidized cytochrome c were obtained from ox heart muscle by the simple method of Keilin & Hartree [1937]. The cytochrome-iron content was estimated[1] by the $\alpha\alpha$-dipyridyl method [Hill & Keilin, 1933].

The experiments were carried out exactly as in the previous sections with 0·1–0·4 ml. of the cytochrome solution added to the medium in the vessels. O_2 uptake rates of various tissues with p-phenylenediamine and with succinate in the presence and absence of added cytochrome c are shown in Table VII.

Effect on p-*phenylenediamine oxidation.* Formerly it was believed that the oxidation of p-phenylenediamine, or the "Nadi" reagent, was catalysed directly by the oxidase, but it will be seen that the oxidation of p-phenylenediamine is greatly accelerated by the addition of cytochrome with all the homogenized

[1] These estimations were kindly made for us by Dr H. K. Alber.

Table VII. *Results of determinations, on the same sample of rat tissue, of the rates of oxidation of succinate and p-phenylenediamine, separately and together, with and without added cytochrome*

Homogenized suspensions used, unless otherwise stated. Activities calculated on moist weights.

Tissue		Cytochrome mg. in 3 ml.	Succ Q_{MB}	$\dfrac{\text{Succ } Q_{MB}}{\text{Succ } Q_{O_2}}$	Succ Q_{O_2}	p-Phen Q_{O_2}	$\dfrac{p\text{ Phen } Q_{O_2}}{\text{Succ}}$
Kidney		—	—	—	22·6	16·4	24·0
		{ —	2·2	0·09	18·9	17·7	26·1
		{ 2·0	—	—	17·4	36·4	34·9
Liver		—	—	—	13·3	10·9	13·1
		{ —	1·8	0·13	13·4	13·2	13·2
		{ 0·7	—	—	13·2	19·1	12·6
		{ 1·4	—	—	—	21·3	···
		{ —	1·4 ±	0·10	13·8	11·0	··
		{ 0·5	—	—	14·4	23·0	14·1
Heart		{ —	—	··	13·0	23·4	26·0
		{ 0·7	— ··	—	24·0	35·5	··
		{ 1·4	—	—	23·8	42·2	43·3
		{ —	3·4	0·28	12·1	16·4	18·1
		{ 1·4	—	—	17·8	43·4	28·6
Brain		{ —	0·81	0·20	4·1	8·4	14·3
		{ 0·7	··	··	4·2	14·9	13·9
		{ —	—	—	4·0	8·9	14·4
		{ 1·4	—	—	4·6	15·7	15·4
		{ 3·5	··	—	—	15·8	···
Testis	Teased	—	··	—	0·94	4·3	3·1
	Homogenized	·	—	··	1·2	2·8	3·8
	Teased	{ ··	—	—	0·69	5·0	3·5
Testis	Homogenized	{ 0·7	··	—	0·73	5·2	4·1
		{ —	0·16	0·09	1·7	3·1	4·8
		{ 0·7	—	—	1·7	6·7	4·8
		{ 1·4	—	—	··	8·3	···
Lung		{ —	0·37	0·42	0·86	1·1	1·2
		{ 2·0	—	—	1·7	6·8	6·0
Skeletal muscle		{ —	0·6	0·32	1·9	2·9	2·6
		{ 1·0	—	—	2·8	10·1	5·7
Spleen		{ —	0·31	3·4	0·09	1·5	1·63
		{ 0·7	—	··	0·85	5·6	2·3
		{ 1·4	··	—	1·3	6·8	·—
		{ —	··	—	0·04	·	···
		{ 0·7	—	—	1·0	··	···
		{ 1·4	—	—	1·7	—	··
Jensen sarcoma		—	0·22	—	2·0	2·9	3·6
		{ —	0·20	0·10	2·0	3·3	4·3
		{ 1·0	··	—	1·9	4·7	4·8
Flexner-Jobling		—	—	—	2·0	1·1	1·3
		{ —	—	···	0·4	0·6	—
		{ 1·6	··	—	1·8	5·0	··
Walker No. 256 carcinoma		{ ··	—	—	0·16	0·51	0·4
		{ 1·6	—	—	1·2	3·8	—
		{ —	0·06	0·7	0·09	0·27	0·37
		{ 0·7	0·06	—	—	0·70	0·52
		{ 1·4	—	···	0·20	0·96	—
Philadelphia No. 1 sarcoma		{ —	0·19	0·48	0·40	0·76	0·79
		{ 1·0	—	—	1·09	4·6	3·3
		{ 2·0	—	—	0·91	5·8	—
		{ —	—	··	0·0	0·65	—
		{ 1·6	··	—	0·8	4·0	—
Pancreas*		{ —	—	—	0·04	0·49	—
		{ 1·6	—	—	0·05	0·48	—

* Results with pancreas are doubtful. It is possible that enzymes are destroyed in suspensions of this tissue. Also the suspensions become acid fairly rapidly.

tissues so far tried: Ogston & Green [1935] found the same with an enzyme preparation from heart. The reduction of oxidized cytochrome c by a solution of p-phenylenediamine occurs immediately and can be readily observed with a hand spectroscope. The Nadi reagent also reduces cytochrome, and with a tissue suspension in an open test tube with added Nadi reagent the development of the blue colour throughout the solution occurs much more rapidly if a little oxidized cytochrome is added. It seems definite, therefore, that these oxidations take place through the mediation of cytochrome, and the oxidation which occurs without added carrier may be due to the cytochrome already present in the tissue suspension. (Keilin & Hartree [1938] have recently demonstrated the same points very thoroughly.)

From Table V it will be noticed that with added cytochrome the rate of p-phenylenediamine oxidation per unit weight of tissue increases toward a maximum with decreasing concentration of tissue and increasing concentration of cytochrome: i.e. the maximum oxidase activity is shown when the ratio of cytochrome concentration to tissue concentration is high. Keilin [1930] showed similar effects of cytochrome and enzyme concentration in the oxidation of cysteine by preparations of heart muscle indophenol oxidase.

It follows that to obtain a maximum estimate of the oxidase content of a tissue it is necessary to add excess cytochrome with the p-phenylenediamine, using low concentrations of tissue. In this way the oxidase activity of rat heart, for instance, is found to be equivalent to the remarkably high figure of $Q_{O_2}=500$ approximately (calculated on the dry weight of tissue). Even these high figures possibly do not represent the absolute maximum for the tissues, since the process of homogenizing probably does not result in the rupture of all cells and the extra cytochrome would not penetrate into intact cells. But it is seen that with every tissue the oxidase activity is considerably higher than is necessary to account for the whole normal respiration as measured by the tissue slice technique. In Table VIII estimates of approximately the full oxidase activities of a few tissues are given.

Table VIII. *Estimates of the full oxidase activities of rat tissues. Rates of O_2 uptake of tissue suspensions in the presence of p-phenylenediamine and excess of cytochrome c*

Tissue	p-Phen Q_{O_2}		Tissue	p-Phen Q_{O_2}	
	On moist wt.	Mean on dry wt.		On moist wt.	Mean on dry wt
Kidney	68·8, 72·0	288	Spleen	6·8, 7·8	32
Liver	51·2	167	Muscle	10·1, 8·1	38
Heart	114·5, 112·0	506	Jensen sarcoma	8·0, 5·7	43
Brain	29·5	134	Flexner-Jobling carcinoma	5·0	28
Testis	13·1	106	Walker No. 256 carcinoma	1·0, 3·8, 1·3	15
Lung	6·8, 7·8	31	Phila. No. 1 sarcoma	4·0, 5·8	29
			Ox retina	10·6	88

With tissue in which the cells have not been extensively broken up by homogenization the results are somewhat different. This is seen (Table VII) in experiments with rat testis which was merely pulled apart into loose bunches of tubules. With this "teased" testis the p-phen Q_{O_2} was higher than with the homogenized tissue but addition of cytochrome scarcely affected the rate. Apparently the oxidation of the p-phenylenediamine takes place inside the cell

where there is a certain concentration of cytochrome; added cytochrome, not being permeable into the cells does not affect the rate. The homogenized tissue has a lower original p-phen Q_{O_2} due, perhaps, to the dilution of the cytochrome from the broken cells, but added cytochrome can now come into contact with the oxidase and so can raise the O_2 uptake rate even above that found in the intact cells with their limited content of carrier.

Effect of succinate oxidation. Results of experiments on the O_2 uptake of tissue suspensions in the presence of succinate with and without the addition of cytochrome are shown in Table VII.

With spleen only a trace of succinoxidase activity was found directly, although the experiments on methylene blue reduction showed the presence of dehydrogenase. It was therefore supposed that the lack of O_2 uptake with succinate was due to lack of carrier, and, as expected, it was found that on the addition of cytochrome the O_2 uptake was increased 10–40 times. With heart there is also a considerable acceleration (50–80 %) of succinate oxidation on adding cytochrome, which shows that, while this tissue contains cytochrome, the amount in a suspension is not enough to carry the rapid H transfer which the high dehydrogenase activity (Succ $Q_{MB} = 3\cdot1, 4\cdot1$) of the tissue is capable of activating.

With brain, testis, kidney and liver the addition of cytochrome does not appreciably affect the succinate oxidation, there being evidently enough cytochrome present to effect H transfer as fast as the dehydrogenase present can "activate" it. It will be noticed that the rate of methylene blue reduction by suspensions of these three tissues is particularly low compared with the rate of O_2 uptake, i.e. the ratio Succ Q_{MB}/Succ Q_{O_2} is particularly low. In any tissue in which this ratio exceeds about $0\cdot2$, it is found that the dehydrogenase activity exceeds the capacity of the cytochrome present to transfer all the H activated; the limiting factor is the concentration of cytochrome (oxidase always being present in excess) and accelerated O_2 uptake is obtained by adding cytochrome. It should be mentioned that experiments showed that the addition of cytochrome had no effect on the time of reduction of methylene blue; no carrier is required when methylene blue replaces O_2 and oxidase.

The rate of O_2 uptake with p-phenylenediamine plus succinate

With a number of tissues the O_2 uptake rate was determined in the presence of optimal concentrations of both succinate and p-phenylenediamine. The term $_{Succ}^{p\text{-Phen}} Q_{O_2}$ is used to express these rates. Results are given in Table VII. It will be seen that with the majority of homogenized tissues, in the absence of added cytochrome, p-phenylenediamine and succinate together gave an O_2 uptake greater than that obtained with either substrate separately. In fact with several tissues, notably brain and testis, the effect was equal to or greater than the sum of the effects of either substrate separately. With other tissues, notably liver, the "additive" effect is not apparent. Battelli & Stern [1912, 1, 2] noticed this "additive" effect with brain and its absence in liver, and considered it probable that the mechanisms for the oxidation of p-phenylenediamine and of succinate were not identical in brain. However, as can be seen in Table VII, the "additive" effect is not confined to brain; it occurs in most tissues, varying from an effect in excess of simple addition of the O_2 uptakes of the two substrates separately, in some experiments with brain, to complete absence of summation with liver. In view of the well-known effects of inhibitors such as HCN, it seems most probable that the oxidations of both p-phenylenediamine and succinate take place almost exclusively through cytochrome and indophenol oxidase. Many

experiments were tried to explain the "additive" effect until it was found that the oxidations of p-phenylenediamine by brain and testis could be accelerated not only by adding succinate but also by fumarate. malonate or acetate, none of which substances was itself rapidly oxidized by the suspensions in the absence of p-phenylenediamine. With liver, fumarate and malonate had little effect on the oxidation of p-phenylenediamine. (Acetate increased the O_2 uptake of liver with p-phenylenediamine markedly. This result was repeatedly observed but cannot yet be explained.) Some results are shown in Table IX.

Table IX. *Effects of Na salts of acids on* p-*phenylenediamine oxidation*

Salt added equivalent to $0·15 M$ Na in each case.

	Cyto-chrome	p-Phen Q_{O_2} on moist wt.				
		No addition	Succinate	Fumarate	Malonate	Acetate
Brain	–	9·8	16·6	14·7	15·3	14·4
	+	23·2	18·0	15·6	16·1	17·4
Testis	–	2·5	3·6	3·2	3·5	3·2
	+	7·4	4·0	3·3	3·4	3·8
Liver	–	11·9	15·0 (16·8)*	12·4	13·9	18·5
	+	40·0	23·9	18·3	19·3	30·9

* Succinate without p-phenylenediamine, i.e. Succ Q_{O_2}.

It seems most likely that the "additive" effect is in reality simply a salt effect of the Na salts of the acids added.

That succinate oxidation is not concerned in the observed stimulation of O_2 uptake of brain and testis with p-phenylenediamine, is shown by the following experiments. (1) Estimations of succinate indicated that, in the presence of p-phenylenediamine, succinate oxidation was inhibited. (Succinate was determined by the manometric succinoxidase method after removing the diamine with permanganate and extraction of the succinate with ether.) (2) Labes & Krebs [1935] and Potter & Elvehjem [1937] showed that selenite in low concentration inhibits succinic dehydrogenase and we have confirmed this both on O_2 uptake and methylene blue reduction. Selenite causes a small, 4–20 %, acceleration of p-phenylenediamine oxidation, and the high O_2 uptake of p-phenylenediamine plus succinate is also slightly further *increased* by selenite. If oxidation of succinate caused the additional O_2 uptake with p-phenylenediamine plus succinate, selenite would *reduce* the total O_2 uptake. Similar results were obtained with malonate which also is known to inhibit succinic dehydrogenase [Quastel & Wooldridge. 1928; Quastel & Wheatley. 1931]. (3) Finally, the formation of dark oxidation products of p-phenylenediamine with brain suspensions is appreciably more rapid when salts of succinate and other acids are added, as can be readily observed in test tube experiments. With liver these salts seem to delay the darkening. The inhibitory effect of the salts on the O_2 uptake with p-phenylenediamine plus cytochrome. mentioned below, can also be observed.

Battelli & Stern [1912, 2] noted that NaCl solutions up to $0·1-0·17 M$ increased the rate of p-phenylenediamine oxidation by brain mince. The succinate (presumably $C_4H_4O_4Na_2$, $6H_2O$) in the concentration used by them [1912, 1] corresponded to $0·12 M$ Na and so should be expected to accelerate p-phenylenediamine oxidation purely as a salt effect; and in fact it did so to just about the same relative extent (31 %, 35 %) as they found with NaCl in their next paper [1912, 2]. With liver mince, weak NaCl solutions, $0·05 M$ increased the rate of oxidation of p-phenylenediamine but with stronger solutions, $>0·1 M$, the rate decreased again. With the amount of sodium succinate used by them it was to be expected that only a small increase in rate of O_2 uptake would be observed.

The mechanism of this salt effect is not clear. It will be noticed (Tables VII and IX) that with all the tissues the O_2 uptake rate with p-phenylenediamine plus excess cytochrome is usually lowered on the addition of succinate or other

salts. The salts may improve the adsorption of cytochrome on the oxidase, in brain and testis particularly, and so increase its rate of oxidation, thus increasing the O_2 uptake rate when cytochrome is the limiting factor; but when excess of cytochrome is present the activity of the oxidase is the limiting factor and salts may have a depressing effect on this. The difference between brain and liver, at the two extremes, may be due to differences in the colloidal behaviour of the tissue particles with which the oxidase is associated. Further work on these points is in progress.

Note. Breusch [1937], in a paper which appeared after the completion of this work, reports a similar distribution of succinoxidase activity, the activity being measured by the formation of fumarate $\rightleftharpoons$ malate. He did not observe the excess of cytochrome oxidase in the tissues.

SUMMARY

1. Using tissues reduced to fine suspensions by Potter & Elvehjem's [1936] method, conditions have been worked out under which the approximate maximum rates of oxidation of succinate and p-phenylenediamine can be observed. A method of applying the Thunberg methylene blue reduction technique for the determination of succinic dehydrogenase in tissue suspensions is described. Using these methods estimates have been obtained of the activity of the complete succinoxidase system, the indophenol oxidase and the succinic dehydrogenase in various tissues.

2. The complete system, "succinoxidase", is extremely active in kidney, liver and heart. Brain, testis, skeletal muscle, lung, adrenals and retina have moderate to low activities. Thymus, pancreas, spleen and blood, have practically no activity. Cancer tissues vary; Philadelphia No. 1 sarcoma, Walker 256 carcinoma (usually) and certain spontaneous mammary carcinomata (rat) are inactive, while Jensen sarcoma, Flexner-Jobling and one example of Walker 256 carcinoma showed definite, though low, activities.

3. The oxidation of succinate by methylene blue is much slower than by O_2, and there is no constant relation in different tissues between the rate of reduction of methylene blue and the rate of O_2 uptake. But in general the tissues fall into the same order when arranged according to their activity measured in either way.

4. The tissues fall into roughly the same order according to their activity in oxidizing p-phenylenediamine.

5. The oxidation of p-phenylenediamine is effected by the indophenol oxidase through cytochrome and it is only in the presence of excess cytochrome that an estimate of the full oxidase activity of the tissues can be obtained. On adding excess of cytochrome c, the oxidation of p-phenylenediamine is considerably increased in all homogenized tissues. In this way it is found that the activity of the tissues is in all cases considerably higher than is necessary to account for the whole respiration of the normal tissue.

Since cytochrome does not penetrate cells, the effect of adding cytochrome is not apparent in incompletely disintegrated tissue.

6. Cytochrome is a necessary link in the complete succinoxidase system. In some tissues, the ratio between rate of methylene blue reduction (i.e. the dehydrogenase estimate) and rate of O_2 uptake is comparatively high. In these cases the O_2 uptake rate with succinate can be increased to a maximum value by adding cytochrome c.

7. In the presence of optimal concentrations of both p-phenylenediamine and succinate, many tissues, notably brain and testis, give O_2 uptake rates

greater than with either substrate separately. This effect seems to be due, not to a difference in oxidases concerned with the two substrates, but to a salt effect of the Na succinate on the oxidase-cytochrome activity; similar results were obtained with fumarate, malonate, and acetate. With liver the "additive" effect is absent. With all the tissues the high rate of p-phenylenediamine oxidation in the presence of added cytochrome is lowered on the addition of succinate and other salts.

8. Blood catalyses the oxidation of p-phenylenediamine strongly but the mechanism is quite different from that in other tissues. The catalyst concerned seems to be a breakdown product of haemoglobin produced by reaction with the diamine. Cytochrome addition has no effect.

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CLXXXVIII. THE RELATION BETWEEN VITAMIN C AND ADRENALINE

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IT was shown by Daoud & Ayyadi [1936] that the rate of glycogenolysis caused by adrenaline, under constant conditions, ran parallel with the degree of saturation of the body with vitamin C. Two alternative suggestions were offered to explain this parallelism. The first was that the degree of saturation of the body with vitamin C determines directly the rate of glycogenolysis, though hyperglycaemia and glycosuria are produced in states of saturation with the vitamin only when the peripheral utilization of the blood sugar is interfered with by adrenaline. The second suggestion was that the vitamin in the reduced form protects adrenaline from rapid oxidation and thus increases glycogenolysis.

As regards the second suggestion, such a relationship has been suggested by different investigators owing to the co-existence of both substances in the suprarenal and to the reduction in the amount of both stated by some to occur in scurvy. The stabilizing effect of the vitamin on adrenaline may be inferred from the work of Szent-Györgyi [1928], Heard & Raper [1933], and Euler & Klussmann [1933]. More recently Heard & Welch [1935], working on the Ringer-Locke perfusate of the suprarenal glands, found that as long as a small amount of reduced ascorbic acid remained in the perfusate there was no diminution in its pressor activity. They also found that the O_2 uptake of the perfusate was characteristic of an oxidation-reduction system. They stated that the perfusate contained neither sulphydryl compounds nor any reducing substances known to occur in the gland other than ascorbic acid, but did contain an agent which minimized the rate of autoxidation of ascorbic acid.

Against the results of these investigators may be set those of Deutsch & Schlapp [1935] who found a reduction in the adrenaline content of the suprarenals of guinea-pigs in inanition but no significant change in their ascorbic acid content, provided that sufficient green food were given in the diet. It thus appeared that inanition was an important factor in the reduction of the adrenaline content of the suprarenals in scorbutic animals. They came to the conclusion that there was no close physiological relation between ascorbic acid and adrenaline.

The relation of these two substances, even in the suprarenal which is rich in both, thus remains obscure. The suprarenal is concerned in the elaboration and storage of adrenaline and it is natural to suppose that it is provided with some system which protects this hormone from destruction; alternatively, it may be deficient in the agents elsewhere responsible for the destruction of adrenaline. This view is strengthened by the fact that the liver is also an organ with a high vitamin C content, yet, according to Markowitz & Mann [1929], Elliott [1905] and Giragossintz & Mackler [1929], the liver is particularly active in the destruction of adrenaline. The work on the suprarenal or its perfusate will

therefore give no clue to the part which the vitamin alone plays in its physiology, especially if we bear in mind, from the data reported by Wiltshire [1931], Kellie & Zilva [1935], Mawson [1935], Hopkins & Morgan [1936] and Heard & Welch [1935], that most of the substances and tissue extracts which exert a protective effect on the vitamin are also protective for adrenaline, and that substances which protect the vitamin are found in the perfusate of the suprarenals. These protective substances are widely distributed in the body, so, even if we admit a protective influence of the vitamin on the adrenaline in the suprarenals or in pure mixtures of the two substances, it will still be questionable whether this protective effect is of particular significance in the body as a whole, where numerous other protective substances are present and where certain organs appear to be more active in the destruction of adrenaline than others.

These considerations made it desirable to find out how far the work done on isolated suprarenals or on adrenaline *in vitro* applied to the functions of vitamin C in the body as a whole and to decide whether the effect of the vitamin in augmenting the rate of glycogenolysis caused by adrenaline is due to a stabilizing effect of the vitamin on adrenaline or is due to a direct glycogenolytic effect of the former.

During the course of this investigation, the effect of the vitamin on the blood pressure was studied in pithed animals.

EXPERIMENTAL

Since experiments carried out *in vitro* or on isolated organs are suspected of being unhelpful as a criterion for the state of events in the body as a whole, it was decided to work on the whole animal. We first compared the effects produced by certain doses of adrenaline injected before and after the gradual increase of the vitamin C content of the body.

A pithed cat was injected intravenously with different doses of 1/10,000 adrenaline hydrochloride solution and the maximum rise in blood pressure and the duration of the rise caused by each dose were recorded. Ascorbic acid (B.D.H.) was then given and the effects of the previous doses of the adrenaline hydrochloride were again tried. Ascorbic acid and adrenaline hydrochloride were then administered alternately to determine the effect of adrenaline after different amounts of ascorbic acid had been administered.

It was noticed that ascorbic acid injected in doses of 100 mg. into a 2700 g. cat had no effect on the blood pressure, but when the dose was increased to 200 mg. a rapid drop in blood pressure occurred. The maximum drop was 24–28 mm. and lasted for 93–95 sec. Reinjection of the same doses of adrenaline which had been given before the administration of ascorbic acid caused a much smaller and less sustained rise in blood pressure than before. Adrenaline injected in considerably larger doses than those given before the ascorbic acid administration caused a rise of much smaller extent and shorter duration than would be expected. Simultaneous administration of the two substances produced the same small effects.

The effect of ascorbic acid in lowering the blood pressure might have been due to its acidity rather than to any specific influence of the vitamin. The observation that adrenaline had less effect on the blood pressure after the ascorbic acid injections might possibly be explained by the fact that the acidity caused the production of substances which antagonized the action of adrenaline. The experiment was therefore repeated on a pithed cat weighing 2·5 kg., the ascorbic acid being neutralized with $N/100$ NaOH immediately before injection. In this experiment the magnitude and duration of the rise in blood pressure

caused by adrenaline were the same after as before the administration of the neutralized ascorbic acid; further, doses of 200 mg. of the neutralized acid caused neither rise nor fall in blood pressure as shown in Table I.

Table I

Substances in the order of injection	Blood pressure (mm. Hg)		Duration of rise or fall sec.
	Maximum rise	Maximum fall	
0·1 ml. adrenaline*	52	—	130
0·2 ,,	96	—	165
0·1 ,,	58	--	130
0·2 ,,	91	—	190
0·1 ,,	54	—	145
0·2 ,,	88	—	165
100 mg. ascorbic acid	—	—	—
100 ,, ,,	—	-	—
200 ,, ,,	—	--	—
0·2 ml. adrenaline	91	—	165
0·1 ,,	56	—	132
100 mg. ascorbic acid	—	—	-
100 ,, ,,	—	—	--
100 ,, ,,	—	--	—
100 ,, ,,	—	—	-
100 ,, ,,	—	--	—
0·1 ml. adrenaline	52	—	135
0·1 ,,	58	—	140
0·2 ,,	89	—	187
0·1 ml. adrenaline + 100 mg. ascorbic acid	57	—	137
200 mg. ascorbic acid	—	—	--
200 ,, ,,	—	--	-
0·2 ml. adrenaline	88	--	190
0·1 ,,	56	--	140
0·1 ,,	53	—	136
0·1 ,,	57	—	145
0·2 ,,	90	--	185

* 1/10,000 adrenaline hydrochloride solution.

The injection of a total quantity amounting to 1·4 g. of ascorbic acid, adjusted to the physiological pH, into a cat of 2·5 kg. body weight neither augmented nor prolonged the effect of adrenaline on the blood pressure.

The above results, although instructive, do not disprove the existence of a protective effect of ascorbic acid on adrenaline, since the destruction of adrenaline in the body appears, from the work of Weiss & Harris [1904], not to coincide with the return of the blood pressure after the injection of adrenaline to its original level. These investigators found that when a cat was injected with adrenaline the transfusion of its blood taken after the blood pressure had fallen to its original level into a second cat caused a rise of blood pressure in the latter. We therefore attempted to find out the time necessary for the destruction of a certain amount of adrenaline in animals saturated with the vitamin and in normal controls in the following manner.

Two cats were chosen of exactly the same weight, 2800 g., and were kept on the same diet. One of the cats received 7 daily injections of 70 mg. ascorbic acid neutralized with Na_2CO_3 immediately before injection. On the 8th day the two cats were anaesthetized with intraperitoneal injections of dial. 5 ml. samples of arterial blood were taken from each cat and injected alternately into a third pithed cat and the small rise in blood pressure was noted in each case.

Each of the two cats was injected 3 times intravenously with 1 ml. adrenaline hydrochloride 1/1000, allowing 5 min. between each injection. Samples of 5 ml. of arterial blood taken after definite intervals of time had elapsed since the last adrenaline injection in each case were immediately injected into the femoral vein of the pithed cat. The rise in blood pressure above the initial rise caused by the blood of each cat before the adrenaline injection was noted in each case. The transfusion of blood was continued till the samples of blood taken from each cat caused no greater rise than that caused by the blood of this particular cat before it had received adrenaline. The time between the last injection of adrenaline and the disappearance of its effect on the blood pressure of the pithed cat was taken as the time necessary for the destruction of adrenaline in the cats under experiment as indicated in Table II.

Table II

Time after last adrenaline injection min.	Rise in blood pressure above initial mm. Hg	
	Control cat	Cat injected with ascorbic acid
5	108	—
20	86	47
40	52	32
60	30	20
80	7	5
90	4	Nil
95	Nil	—

It will be noticed that in this experiment the rise in the blood pressure of the pithed cat caused by the blood of the cat injected with ascorbic acid was less than in the case of the control cat, but this was not always so. The main observation is that the time taken by the two cats to destroy the same amount of adrenaline is practically the same, being, in fact, 5 min. less in the case of the cat previously injected with ascorbic acid. In another experiment a total dose of 2 ml. of 1/1000 adrenaline hydrochloride was injected into each of two cats weighing 2700 g.; the destruction of adrenaline took place in 70 min. in both cases and there was no appreciable difference in the magnitude of rise of blood pressure caused by the two bloods. These two experiments are especially recorded because the blood taken from the two cats before adrenaline injections did not show a depressor effect on the pithed cat, as sometimes happened, and the experiments were successfully carried out to the end.

The above result seems to indicate that, in the intact animal, ascorbic acid has no protective effect on adrenaline. Since it has been shown by Sandiford [1920] and by Cori & Cori [1928] that adrenaline causes a rise in metabolism, it was decided to compare the time necessary for the disappearance of the effect of adrenaline on the metabolism of rats saturated with ascorbic acid with the time necessary in the case of unsaturated rats. For this purpose a number of male rats were chosen weighing 250–255 g. They were divided into two batches and all were kept on a scorbutic diet for 7–10 days. Each of the members of one batch then received daily injections of 7 mg. of ascorbic acid for 3–9 days before the adrenaline administration. Food was removed at 4 p.m. on the day preceding an experiment. On the days of experiments, one pair of rats taken from each batch was injected with 0·1 ml. physiological saline and the O_2 consumption by the pair was then determined every 20 min. in a closed system.

In the first 20 min. a high consumption was always noticed, which was undoubtedly due to the effect of handling; the observations were continued until steady consumptions were attained in the subsequent 20 min. periods. Usually the amount of O_2 consumed in the second or third 20 min. agreed with the subsequent readings. The constant amount of O_2 consumed was taken as the basal amount for the pair in 20 min. Each rat then received an injection of 0·1 ml. 1/1000 adrenaline hydrochloride solution and the readings were again taken until the quantity of O_2 consumed in 20 min. returned to the basal amount. The time between the adrenaline injection and the end of the last 20 min. during which there was still a rise in O_2 consumption above the basal, was taken as an estimate of the time necessary for the wearing away of the effect of a definite amount of adrenaline in the bodies of the saturated and unsaturated rats. In a very few cases, continuous fluctuations in the O_2 consumption in the 20 min. periods were noted, especially after adrenaline injections, but such results were rejected. Many experiments were performed in the manner described; for the sake of brevity, the results obtained with six pairs of rats are given in Table III.

Table III

	Time for disappearance of adrenaline effect (min.)					
Diet	I	II	III	IV	V	VI
Scorbutic	140	120	160	140	120	120
Scorbutic + ascorbic acid injections	120	120	140	140	160	140

The time necessary for the disappearance of the effect of the adrenaline in the two groups was of the same order and varied from 120 to 160 min. This result agreed with that of the blood transfusion experiments in suggesting that ascorbic acid had no protective effect on adrenaline *in vivo*.

It has already been mentioned that Daoud & Ayyadi [1936] gave two alternative suggestions in explanation of the parallelism between the degree of saturation of the body with vitamin C and the rate of glycogenolysis caused by adrenaline. The present work rules out the suggestion of a protective effect of vitamin C on adrenaline.

Effect of vitamin C on adrenaline in vitro

Adrenaline is known to undergo rapid autoxidation in atmospheric oxygen. Although there is a tendency at present to consider that adrenaline is destroyed in the body by enzymes, yet the rapidity of autoxidation suggests at least that this may be partially responsible for the destruction. This, together with the apparent similarity between the mechanisms of autoxidation and some of the enzymic oxidations, made it desirable to examine the effect of the vitamin, when isolated from other systems, on the oxidation of adrenaline.

Heard & Welch [1935], in their work on the effect of atmospheric oxygen on the perfusate of the suprarenals already referred to, concluded that the stabilization of adrenaline was effected by means of an oxidation-reduction system, and that ascorbic acid in the reduced form was the agent responsible for the protection of the catechol group of adrenaline from oxidation to o-quinone. When the ascorbic acid was completely oxidized, irreversible oxidation of adrenaline, manifested by the appearance of a red coloration and diminution in pressor activity, commenced; thereafter the rate of adrenaline oxidation was dependent on the amino-acid content of the perfusate.

It was planned to study the problem in solutions containing mixtures of the two pure substances. Since adrenaline is more stable in acid than in neutral or alkaline solutions, the effect of the acidity of ascorbic acid was eliminated by buffering the solutions at pH 7 with phosphate. The relative proportions of adrenaline hydrochloride and ascorbic acid in the solutions were of the order reported by Heard & Welch to be present in some of the perfusates of the suprarenals; the ratio of ascorbic acid to adrenaline was increased in some of the experiments for the purpose of comparison.

It is generally accepted that the appearance of the red colour in adrenaline solutions is an indication of irreversible oxidation of adrenaline and is accompanied by diminution in pressor activity. The red-coloured substance was considered by Green & Richter [1937] to be adrenochrome, which is inactive as a vasoconstrictor.

In the present work the commencement of irreversible oxidation was judged at first by the appearance of this red colour. Equal volumes of a solution of adrenaline hydrochloride in phosphate buffer were placed in a number of bottles of 250 ml. capacity. The bottles were all brown in colour with the exception of one colourless bottle which was used for comparing the effect of light on the decomposition of adrenaline. The total volume of solution in each bottle was always 100 ml.; all solutions were buffered at pH 7 and contained the concentrations of adrenaline alone or adrenaline and ascorbic acid indicated in Table IV. In addition, control experiments were made in which only buffered ascorbic acid (B.D.H.) was included. All bottles were tightly stoppered with glass stoppers and were shaken every now and then for the same length of time on a mechanical shaker to facilitate oxidation by the air enclosed above the liquid. After each period of shaking the brown bottles were placed in the dark and the colourless bottle was placed in the light.

In most of the experiments the reddish tinge appeared in the different solutions in the following order: adrenaline alone kept in light, simultaneously in the different solutions of adrenaline + ascorbic acid, adrenaline alone kept in dark; no red colour appeared in the solutions containing ascorbic acid alone. In some cases, however, the appearance of the red tinge in the solutions containing adrenaline and ascorbic acid together took place at the same time as in the solutions of adrenaline alone kept in the light (Exp. 2, Table IV). There was

Table IV

Comparative intensities of reddish colour

| | | | In coloured bottles in dark | | |
Exp.	Time of observation hr.	In light 2 mg. adr.	2 mg. adr.	2 mg. adr. +1 mg. asc.	2 mg. adr. +2 mg. asc.	2 mg. adr. +4 mg. asc.
1	3	Trace	−	−		
	24	+	−	−		
	48	+ +	−	+		
	72	+ +	−	+ +		
	96	+ +*	+	+ +*		
2	20	+	−	+	+	
	44	+ +	+	+ +	+ +	
3	24	Trace	−	−	−	−
	48	+	−	−	−	−
	72	+ +	−	+	+	+
	96	+ +	+	+ +	+ +	+ +*
	110	+ +*	+ +	+ +*	+ +*	+ +*

* Yellow tint.   adr. = adrenaline hydrochloride.   asc. = ascorbic acid.

no definite rule as regards the time of first appearance of colour in the solutions kept in the light. The rate at which the reddish colour appeared and increased in intensity in the different solutions is indicated by the results of 3 experiments recorded in Table IV.

It is clear that light accelerates the coloration of adrenaline solutions and that when the effect of this factor is eliminated the solutions containing adrenaline and ascorbic acid become coloured earlier than similar solutions containing no ascorbic acid. If the coloration of adrenaline solutions is accepted as an indication of oxidative destruction, this result would mean an acceleration of the destruction of adrenaline by ascorbic acid. When, however, the pressor activity of the solutions was actually tested, a contradictory result was obtained.

Solutions were prepared as before, some being made up one day before the others, for the purpose of studying the correlation between the intensity of colour and the pressor activity. The pressor activity of all the solutions was compared with that of a freshly prepared adrenaline hydrochloride solution buffered at pH 7 by measuring their effect on the blood pressure of one and the same pithed cat. Equal doses of 0·5 ml. were injected. The results of a number of such experiments are given in Table V.

Table V

Soln. no.	Contents of soln. mg. per 100 ml.	Condition	Age of preparation days	Intensity of colour	Mean rise in blood pressure mm. Hg	Strength relative to fresh soln. adr.
1	2 adr.	In light	3	+ +	28	0·25
2	2 adr.	In dark	3	+	40	0·36
3	2 adr.	In light	2	+	40	0·36
4	2 adr.	In dark	2	-	54	0·48
5	2 adr. + 1 asc.	In dark	2	+	54	0·48
6	2 adr. + 2 asc.	In dark	2	+	54	0·48
7	2 adr. + 4 asc.	In dark	2	+	54	0·48

adr. = adrenaline hydrochloride.   asc. = ascorbic acid.

Observations obtained after allowing the solutions to stand more than 3 days are not recorded owing to complications which occurred after this period, such as the development of a yellow tint.

It can be seen that under the specified conditions of concentration and pH all solutions, whether coloured or not, were less active than a similar but freshly prepared adrenaline solution. Even the colourless solution (4) lost some of its pressor activity. According to Ball & Chen [1933] the first oxidation product, the o-quinone compound corresponding to adrenaline, is not coloured. This is supported by the work of Raper [1926; 1927] on the oxidation of the related substance dihydroxyphenylalanine. If we assume that in solution (4) a complete or partial transformation into the o-quinone compound did take place, it follows that autoxidation of the OH groups of the catechol ring results in the formation of a compound of lower activity than the original adrenaline. The comparison of (4) with (3) does not support the view that the o-quinone compound is quite devoid of activity.

In the solutions containing adrenaline alone at pH 7, the earlier production of colour in the light than in the dark was accompanied by greater diminution in pressor activity (3, 4). The further increase of colour intensity was accompanied by further loss of pressor activity (1, 2, 3). Although there was an earlier development of colour in the light than in the dark, when the intensity of coloration became the same in both cases the solutions had the same activity (2, 3).

According to Green & Richter [1937], the red-coloured substance adreno-chrome is produced by further oxidation of the primary o-quinone product of adrenaline oxidation and involves the removal of one hydrogen atom from the —NH— group of the side chain and one from the o-quinone ring, with the formation of an indole ring. According to them the first oxidation product is extremely unstable and at pH 7 undergoes an almost instantaneous change into the red-coloured adrenochrome which is physiologically inactive. The association of colour production with the formation of an indole ring from the o-quinone derivative of dihydroxyphenylalanine has also been pointed out by Raper [1926; 1927]. The red-coloured adrenochrome, therefore, is the second product of autoxidation of adrenaline although it constitutes the first visible stage in its oxidation. It is clear from the present work that this latter stage is photo-catalysed, since there is an earlier development of colour in solutions of adrenaline alone kept in the light than in solutions prepared at the same time but kept in the dark, in which the original adrenaline proved to have undergone a change which is responsible for most of the total lowering of activity. This explains the further diminution in pressor activity as the colour develops (3, 4) and the proportionality of this diminution to the intensity of coloration (1, 2, 3, 4). It also explains why, when the intensity of coloration of solutions kept in the light and in the dark becomes the same, they have the same pressor activity, for the amount of active and inactive substances at this point will be the same in both cases.

Although there was an early development of colour in the solutions of adrenaline containing ascorbic acid, these showed an activity similar to that of a colourless solution prepared at the same time and kept under the same conditions, and a greater activity than a coloured solution also prepared at the same time but kept in the light. When the solutions 4–7 (Table V) were allowed to stand for another day, nos. 5–7 containing ascorbic acid developed maximum red colour and solution 4 developed a slight coloration; but while the pressor activity of nos. 5–7, when again compared with that of a freshly prepared solution, kept constant as before, that of no. 4 had diminished. These experiments show that ascorbic acid at pH 7 has a limited protective effect on the activity of adrenaline. This limited effect was still more prominent when 1 in 10^6 solutions of adrenaline alone and adrenaline containing the same amount of ascorbic acid were allowed to oxidize in the presence of air and light: it was found that after 24 hr. the solutions containing adrenaline alone completely lost their activity, while those containing ascorbic acid were still active, though they had a lower activity than freshly prepared solutions of the same concentration and reaction. The inactivation of solutions of adrenaline alone, in the light or in the dark, is proportional to the colour development, but no such proportionality exists in the solutions containing ascorbic acid as well. In the latter solutions, both the development of colour and the preservation of activity are independent of the amount of ascorbic acid present.

We can find no explanation of these experimental facts except by assuming (1) that the red-coloured product obtained with solutions of adrenaline con-taining ascorbic acid is different from the inactive adrenochrome produced in solutions of adrenaline alone; it might result from a reaction between the o-quinone primary oxidation product and ascorbic acid; its association with colour is an indication that it would also be an o-quinone: (2) that the protective effect of ascorbic acid hinders the transformation of the o-quinone primary oxidation product of adrenaline into the inactive adrenochrome. These assump-tions explain (1) the preservation of an activity equal to that of the colourless

primary oxidation product by the solutions containing adrenaline and ascorbic acid, (2) the earlier appearance of coloration in these solutions than in those of adrenaline alone under the same conditions, (3) why further increase in colour intensity can take place without further loss of pressor activity, contrary to what occurs in solutions of adrenaline alone, since the increased intensity in the presence of ascorbic acid may simply mean the transformation of the colourless o-quinone into a coloured one of the same activity. According to Green & Richter [1937] ascorbic acid prevents the catalytic oxidation of adrenaline to adrenochrome.

Modified dichlorophenol-indophenol titrations showed that at pH 7 the rate of disappearance of the reduced form of ascorbic acid was rapid and was the same in solutions containing adrenaline as in similar concentrations of ascorbic acid alone. Complete disappearance took place in a few hours in both cases. The accelerated appearance of coloration in solutions containing adrenaline and ascorbic acid cannot therefore be due to the reduced form of ascorbic acid since the latter disappears long before the appearance of colour; the oxidation products of ascorbic acid may be responsible. These observations prove that the continued presence of the reduced form of ascorbic acid itself is not essential for the maintenance of the activity previously indicated to take place in the solutions of adrenaline with ascorbic acid, since it has been shown that the preservation of an activity equal to that of the primary oxidation product can continue after the development of the colour and, as can be seen, after the disappearance of the reduced form of ascorbic acid. Whether the coloured substance formed in the solutions which had contained ascorbic acid is concerned with the preservation of activity remains to be investigated.

The loss of potency in adrenaline solutions containing ascorbic acid (indicated in Table V) when compared with a freshly prepared solution of adrenaline alone was shown by further experiments to be demonstrable even before the complete disappearance of the reduced form of ascorbic acid, while at pH 7 a freshly prepared solution of adrenaline containing ascorbic acid had the same potency as a freshly prepared solution of adrenaline alone. It appears, therefore, that at pH 7 there is at first a simultaneous oxidation of the reduced form of ascorbic acid and of adrenaline which is changed to the corresponding o-quinone, thereafter there is a protection of the o-quinone compound from further oxidation.

These in vitro experiments were concerned with the protective effect of ascorbic acid on the pressor activity of adrenaline when the former was deprived of its acidic properties. In such experiments there is a rapid oxidation of ascorbic acid; a better protective effect on adrenaline might be expected in animal tissues, owing to their tendency to keep ascorbic acid in the reduced form.

The catalytic oxidation of adrenaline to the inactive adrenochrome was shown by Green & Richter [1937] to take place in the animal body by (1) a cyanide-insensitive system present in heart and skeletal muscle and (2) the cytochrome-indophenol oxidase present in all tissues. In the present work, as well as in the experiments of the above investigators, the hindrance of adrenochrome formation by ascorbic acid is indicated. In the intact animal, even if an increased concentration of ascorbic acid is effective in hindering adrenochrome formation, it is evident from the present work that the influence of this factor is combated by other factors, so that the same destruction of adrenaline is produced in normal animals as in those saturated with ascorbic acid. The possibility of adrenaline destruction in the body by mechanisms other than adrenochrome formation can be imagined from the different modes of adrenaline oxidation reported by previous investigators. Thus Weinstein & Manning [1937]

found a substance having the properties of protocatechuic acid in the urine of rabbits in which adrenaline had been injected, which indicates an oxidative removal of the side chain of the adrenaline molecule. Blaschko *et al.* [1937] have reported the presence of adrenaline oxidase in mammalian liver and other tissues; this was subsequently found by Richter [1937] to be an amine oxidase which induced an oxidative deamination in the side chain with the production of an aldehyde and methylamine.

It seems that any interference with the side chain of the adrenaline molecule by oxidative splitting or closure to form an indole ring results in complete loss of pressor activity, while autoxidation of the hydroxyl groups in the catechol ring simply lowers the activity.

SUMMARY

1. Small doses of unneutralized vitamin C injected into a pithed cat have no effect on the blood pressure, but large doses have a depressor effect. When the vitamin is adjusted to the physiological *p*H it has no action. The effect of the large doses of the unneutralized vitamin is ascribed to its acidity rather than to its real influence.

2. At the physiological *p*H, vitamin C augments neither the magnitude nor the duration of the adrenaline effect on the blood pressure, but the acidic vitamin diminishes both its magnitude and its duration.

3. The destruction of adrenaline is the same in normal intact animals as in those saturated with vitamin C. Any protective effect of the latter on adrenaline is combated by other destructive factors operating in the organism.

The parallelism previously observed between the rate of glycogenolysis caused by adrenaline and the degree of saturation of the body with vitamin C cannot be ascribed to a prolongation of the effect of the former through a protective effect of the latter.

4. *In vitro* at *p*H 7 ascorbic acid has a limited protective effect on the pressor activity of adrenaline. It appears that this protection consists in a temporary hindrance of further autoxidation of a primary oxidation product of adrenaline which has a lower pressor activity than the original compound. It is suggested that this primary product is the colourless *o*-quinone corresponding to adrenaline. The continued presence of the reduced form of ascorbic acid is not essential for this protection.

5. The fact that coloration occurs earlier in mixtures of ascorbic acid and adrenaline than in solutions of the latter alone cannot be taken as a sign of earlier destructive oxidation as in the case of adrenaline alone; it may be due to some sort of reaction between the primary oxidation product of adrenaline and the products of ascorbic acid oxidation. The coloured substance produced in these mixtures is suggested to be an *o*-quinone possessing a pressor activity equal to that of the colourless *o*-quinone compound.

6. The effect of light on the destruction of adrenaline appears to be concerned particularly with hastening the second step of autoxidation which is associated with the development of colour.

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CLXXXIX. CARBOHYDRATES IN PROTEINS

I. THE CARBOHYDRATE COMPONENT OF CRYSTALLINE EGG ALBUMIN

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THE question of the occurrence of carbohydrate groups as essential parts of the structure of proteins (apart from mucins and mucoids) has been the subject of many investigations, none of which however has yielded an unequivocal result.

Before the peptide theory of proteins was generally accepted, numerous papers were published concerning the carbohydrate content of proteins. Pavy [1893] hydrolysed coagulated egg-white first with alkali and then with dilute acid and obtained glucosazone from the hydrolysate. He assumed that all proteins contain fairly large amounts of carbohydrate and that the preformed sugar in the protein molecule is responsible for the conversion of protein into carbohydrate which occurs in the animal body. These contentions were the subject of a heated controversy and, although Pavy's physiological hypotheses were somewhat bizarre, his chemical observations were correct. Eichholz [1898] confirmed Pavy's findings by showing that egg albumin, from which mucin and mucoid had been carefully removed, gave on hydrolysis a substance which reduced Fehling's solution and from which an osazone of M.P. 208° could be obtained. Later, when crystalline egg albumin had been prepared, Hofmeister [1898] and Langstein [1902] obtained an osazone from the hydrolysate of the crystalline protein and Seemann [1900] isolated glucosamine hydrochloride from a thrice crystallized sample. The amounts isolated, however, were very small and it was generally assumed that ordinary proteins are composed of amino-acids only and that the presence of sugars in hydrolysates is due to impurities such as mucoids (see Levene [1923] and Plimmer [1917]).

Much later, the question was taken up again by Fränkel & Jellinek [1927] who hydrolysed apparently non-crystalline egg albumin with baryta, removed amino-acids with neutral lead acetate and precipitated the polysaccharide with ammoniacal lead acetate. They repeated this separation several times and finally obtained, by precipitation with alcohol, an optically inactive polysaccharide. This substance on acid hydrolysis gave mannose and glucosamine. Levene & Mori [1929], following the work of Fränkel & Jellinek, introduced as an additional step in the purification of the polysaccharide the use of mercuric sulphate for the removal of nitrogenous impurities. These authors, however, obtained only small amounts of polysaccharide from repeatedly recrystallized egg albumin and concluded therefore that the carbohydrate is not derived from the egg albumin itself but from ovomucoid. Rimington [1929; 1931] applied the same method of hydrolysis and isolation to mixed serum proteins and also obtained mannose and glucosamine hydrochloride as ultimate hydrolysis products.

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Sørensen [1934], using a colorimetric technique, detected carbohydrates in nearly all proteins examined by her, and estimated that crystalline egg albumin contains 1·8 % of mannose.

There remains therefore considerable doubt as to whether crystalline proteins do in fact contain carbohydrates as part of their molecular structure. The positive colour reactions for carbohydrates which are given by highly purified albumin preparations are not sufficient to establish the point with certainty, whilst the failure of workers such as Levene & Mori to obtain more than traces of carbohydrate from purified proteins may well be due to defects in the methods employed.

Thus, prolonged hydrolysis with strong alkali as used by Levene & Mori is well known to be destructive of many carbohydrates and it will in fact be shown later in this paper that such treatment is harmful to the polysaccharide obtained from egg albumin. Moreover, it can hardly be claimed that the method used by these workers for the isolation of the carbohydrate, depending as it does on quantitative removal of amino-acids by neutral lead acetate and mercuric sulphate and selective precipitation of the polysaccharide by ammoniacal lead acetate, is well adapted to the separation of a small amount of carbohydrate in a pure state from a protein hydrolysate.

The object of the work to be described in the present paper has been first to develop a new method for the isolation of carbohydrates from proteins which should be free from the objections mentioned above and secondly to apply the new method to the study of what is perhaps the best-defined protein at present available, namely crystalline egg albumin.

Attempts to separate carbohydrate from egg albumin by physical means

The possibility of the separation of carbohydrate from crystalline proteins by physical means was first investigated, and for this purpose the carbohydrate content of crystalline egg albumin was estimated by the orcinol method after successive recrystallizations, after denaturation and after ultrafiltration. The justification for the use of a colorimetric method for the estimation of carbohydrate will appear later from the fact that the amount of sugar finally estimated by actual isolation agrees satisfactorily with the colorimetric value.

Table I. *Change of carbohydrate content with recrystallization*

Crystallization no.	% Carbohydrate
1	2·8
2	2·0
3	1·8
4	1·75
5	1·8
6	1·8
7	1·8

Table I shows that the carbohydrate content of crystalline egg albumin falls during the first two recrystallizations but is not affected by further repetitions of the process. The final value of 1·8 % carbohydrate calculated as mannose obtained agrees well with that recorded by Sørensen [1934]. Crystallization in itself therefore, so far from effecting a separation of carbohydrate from the protein, leads to a product of constant carbohydrate content.

An attempt was then made to separate the carbohydrate from the protein by ultrafiltration through filters of known porosity. Table II shows that a membrane of average pore diameter 5·5 mμ, which retains egg albumin completely

[Elford & Ferry, 1936], is also impermeable to the carbohydrate associated with it; moreover, for membranes of intermediate porosity the ratios of carbohydrate and nitrogen in the ultrafiltrates are constant within the limits of experimental error. It is clear therefore that the carbohydrate is associated with particles of molecular size similar to that of egg albumin.

Table II. *Ultrafiltration of egg albumin*

Protein	Average pore diameter of membrane in $m\mu$	Carbohydrate content		Protein content		Carbohydrate content of protein	
		Supernat.	Ultrafiltr.	Supernat.	Ultrafiltr.	Supernat.	Ultrafiltr.
		mg. per ml.		mg. per ml.		%	
Native	35	0·324	0·321	1·786	1·714	1·80	1·86
,,	11	0·424	0·095	2·370	0·452	1·77	2·10
,,	9	0·464	0·043	2·740	0·224	1·70	2·00
,,	5·5	0·545	0·010	3·260	0·003	1·70	—
Acid denat.	15	0·325	0·010	1·820	—	1·77	—
Heat denat.	15	0·039	0·060	0·207	0·010	1·88	—

Finally Table II also shows that no carbohydrate is split off on denaturation with acid. The fact that the carbohydrate, which passes readily through the $15\,m\mu$ membrane when combined with the native protein, is completely retained with the protein after the latter has been denatured is convincing evidence that the carbohydrate must be associated with a denaturable protein and not, for instance, with contaminating mucin. This is confirmed by the fact that no carbohydrate is liberated during denaturation by heat.

The impossibility of separating carbohydrate from recrystallized egg albumin by any of the physical processes described affords reasonable ground for the assumption that the carbohydrate forms an integral part of the protein molecule. With respect to denaturation the possibility remains that the carbohydrate group may play some part in the intramolecular mechanism of this process while remaining attached to the protein molecule.

New method for the isolation of carbohydrate from protein hydrolysates

From the experiments of Rimington [1931], it can be deduced that trypsin does not hydrolyse the polysaccharide derived from serum proteins. It is reasonable to expect that the carbohydrate in egg albumin will be similarly resistant and hydrolysis by trypsin was therefore chosen for the present work. Introduction of extraneous carbohydrate with the trypsin was minimized by keeping the amount of enzyme as low as possible; since the (colorimetric) carbohydrate content of the trypsin preparation used was very small, the amount of carbohydrate introduced with the enzyme was actually less than 0·2 % of the total.

The new method of isolation of the carbohydrate from the hydrolysis mixture was based on the following considerations. Ketene reacts quantitatively with α-amino-acids (and α-imino-acids) [Neuberger, 1938] in aqueous solutions of neutral and alkaline reaction to form N-acetyl compounds [Bergmann & Stern, 1930]. In the basic amino-acids the amino groups only are acetylated; thus the action of ketene on histidine and arginine yields α-N-acetylhistidine and α-N-acetylarginine respectively; these acetylated acids retain a zwitterion structure, and are therefore insoluble in organic solvents. Aliphatic hydroxyl groups on the other hand are not acetylated by ketene in aqueous solution, as shown by the fact that ketene reacts with glucosamine to form N-acetylglucosamine only

[Bergmann & Stern, 1928]. It is to be expected therefore that if a mixture of amino-acids and carbohydrate is treated with ketene, all amino-acids except histidine and arginine will be converted into compounds which are soluble in organic solvents, whereas carbohydrates will remain unchanged, apart from acetylation of the amino group of glucosamine or other amino-sugars.[1]

By treatment of a tryptic hydrolysate of protein with ketene and subsequent exhaustive extraction of the acidified solution with chloroform, after removal of basic amino-acids with phosphotungstic acid, it should therefore be possible to remove a large proportion of nitrogenous material without affecting the carbohydrate.

Now carbohydrates in general are readily and completely acetylated by acetic anhydride and pyridine; treatment of the material remaining in the chloroform-extracted solution with these reagents should therefore convert the carbohydrate into a chloroform-soluble condition in which it can easily be separated from the remaining nitrogenous contaminants. It should then be possible to deacetylate the chloroform-soluble acetylated carbohydrate under mild conditions, and to isolate the free carbohydrate by precipitation with alcohol.

A difficulty might arise in the application of the procedure just outlined owing to the formation of chloroform-soluble acetaminoketones by the reaction studied by Dakin & West [1928]; actually it has been shown [Neuberger, 1938] that if the temperature of the acetic anhydride-pyridine mixture is kept low such formation of acetaminoketones from amino-acids is almost entirely suppressed in favour of the formation of acetamino-acids; in any case, even if small amounts of acetaminoketones and/or acetamino-acids should pass into the final chloroform extract, these will be unaffected by the deacetylation process and, being readily soluble in alcohol, will remain in the mother liquor when the free carbohydrate is precipitated.

Table III. *Distribution of nitrogen (N) and carbohydrate (CH)*
during process of fractionation

Tryptic hydrolysate (N, 18·80 g.; CH, 2·16 g.)

Pptn. | Phosphotungstic acid

Ppt. | 50 % acetone   Soln. (N, 14·2 g.; CH, 2·1 g.)

Soln.   Ppt.   Ketene | CHCl$_3$ extraction
(N, 3·2 g.; CH, 50 mg.) (N, 1·4 g.; CH, 10 mg.)

Aqueous | soln.   CHCl$_3$ extr.
         (N, 7·8 g.; CH, 2·1 g.)   (N, 6·4 g.; CH, zero)

Evap. | Pyridine + Ac$_2$O

Water-soluble   CHCl$_3$-soluble
(N, 7·65 g.; CH, 0·8 g.)   (N, 0·105 g.; CH, 1·26 g.)
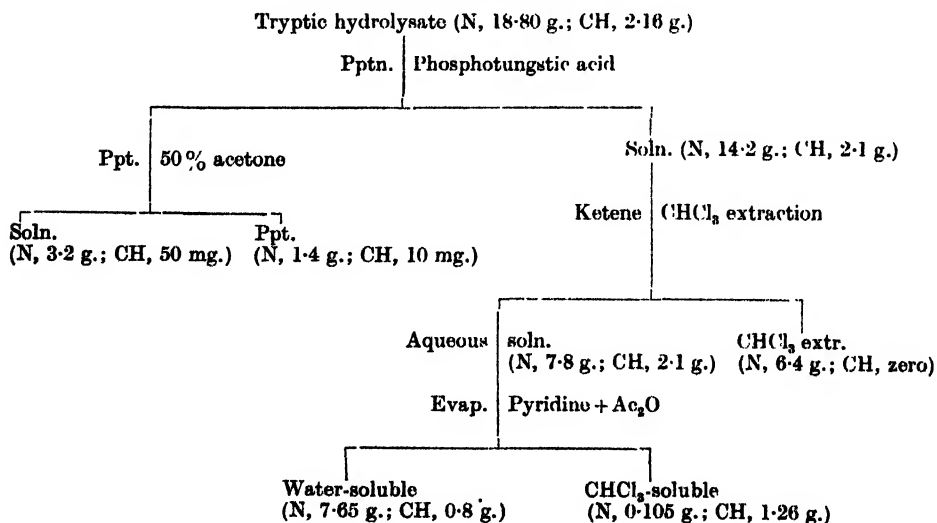
[1] The differential extraction of acetamino-acids by organic solvents as a means of separating amino-acids is being examined by Mr R. Ll. M. Synge in the Biochemical Laboratory, Cambridge.

The procedure finally adopted for the isolation of the polysaccharide has been as follows. The hydrolysate is first treated with phosphotungstic acid to remove basic amino-acids and incompletely digested protein. The material not precipitated by phosphotungstic acid is then treated with ketene, and acetylated amino-acids are removed so far as possible by chloroform extraction. The material obtained on evaporating the aqueous solution is treated with pyridine and acetic anhydride at 0°. The acetylated polysaccharide thus obtained is then extracted from the aqueous solution of the reaction product by chloroform and carefully deacetylated by dilute alkali in the cold, and the free carbohydrate is precipitated by addition of alcohol.

Table III shows the distribution of nitrogen and carbohydrate in the different fractions in a typical experiment; it will be seen that very little carbohydrate is lost by precipitation with phosphotungstic acid and no carbohydrate at all passes into the first chloroform extract. It is clear from Table III that the partition coefficients of some acetamino-acids between chloroform and water are not very favourable for their complete removal from the aqueous phase.

It also appears from Table III that only 60 % of the polysaccharide is obtained in the second chloroform extract after one treatment with pyridine and acetic anhydride. In later experiments it was found that 95 % of the total carbohydrate could be obtained in the final alcohol precipitate by repeating the acetylation process on the residue.

Properties of the carbohydrate

General. The substance obtained, having the appearance and general properties of a polysaccharide, was homogeneous, since samples obtained by fractional precipitation with alcohol had identical rotations, molecular weights and nitrogen and carbohydrate contents (Table IV). Colour reactions indicated

Table IV. *Physical and chemical properties of different preparations and fractions obtained by fractional precipitation with alcohol*

	Nitrogen content %	$[\alpha]_D$	Mol. wt. uncorr. for ash	Carbohydrate content %
Preparation 1	4·85	+ 21·5	1150	47·0
Preparation 2	4·95	+ 22·0	—	48·0
Preparation 3	5·05	+ 22·0	—	50·0
Preparation 4	4·95	+ 22·5	1130	48·0
Fraction 1 precipitated by 50 % alcohol	4·85	+ 22·0	—	46·5
Fraction II precipitated by 65 % alcohol	4·90	+ 20·5	1160	50·1
Fraction III precipitated by 75 % alcohol	5·10	+ 22·5	1110	49·0
Fraction IV precipitated by 90 % alcohol	5·15	+ 22·0	—	—

the presence of hexose and acetamino-sugar, whilst the presence of significant amounts of ketoses, pentoses and uronic acids could be excluded. The substance gives the ninhydrin reaction after acid hydrolysis; this does not necessarily imply the presence of amino-acids however since glucosamine hydrochloride gives a typical ninhydrin test. The negative biuret test showed that the polysaccharide was not contaminated by higher peptides. Negative colour reactions further indicated the absence of tyrosine, tryptophan, histidine, arginine and cystine from the preparation.

Component sugars. The polysaccharide itself had no free reducing group, but reducing sugars were liberated on acid hydrolysis. Fig. 1 shows that the maximum reducing value on hydrolysis with 1·5N HCl at 100° is obtained after about 5 hr.

If correction is made for the "salt error" and the slightly higher reducing equivalents of mannose and glucosamine as compared with glucose, the amount of reducing sugar in the polysaccharide can be calculated to be about 75 %. On the other hand the amount of sugar other than amino-sugar, as determined colorimetrically, was about 48 %, a figure nearly identical with that obtained for the fermentable sugar. Thus, about 50 % of the polysaccharide or 68 % of the reducing sugars is present as fermentable hexose. On the other hand direct colorimetric estimation of amino-sugar indicated a content of 25 % calculated as glucosamine [Elson & Morgan, 1933]. Thus, it can be concluded that the reducing sugar obtained on hydrolysis consists of two-thirds of a fermentable hexose and one-third of an amino-sugar.
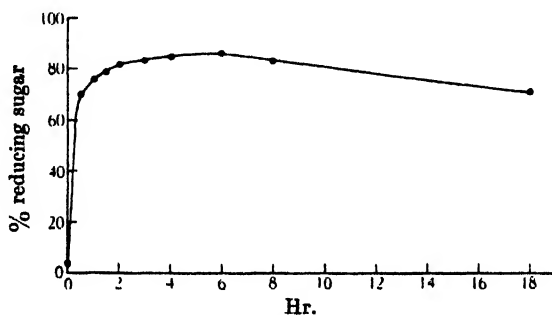
Fig. 1. Liberation of reducing sugar on hydrolysis of polysaccharide with 1·5 N acid at 100°.

Mannose was isolated from a hydrolysate of the polysaccharide in the form of the phenylhydrazone and the p-bromophenylhydrazone. The amounts corresponded to 36 and 38 % of the polysaccharide, whilst in model experiments under the conditions used for the isolation of the hydrazones, mannose could be recovered to the extent of 75–80 %. If such a correction for the incomplete recovery of mannose is applied to the values obtained, the content of mannose in the polysaccharide falls between 45 and 48 % which is in satisfactory agreement with the values obtained from the fermentable reducing sugar content and from colorimetric determination.

Glucosamine was isolated by a newly devised method as 2:4-dihydroxy-benzylideneglucosamine. The amounts isolated in different experiments corresponded only to about 60 % of the value found colorimetrically. The presence of an amino-sugar other than glucosamine can therefore not be excluded with complete certainty.

Nitrogenous components other than amino-sugars. The maximum content of amino-sugar (calculated as glucosamine) accounts for no more than 40 % of the total nitrogen of the polysaccharide; on the other hand, after acid hydrolysis under conditions which would not destroy amino-sugars, 60–70 % of the total nitrogen appears as amino-nitrogen (Van Slyke) and about 20 % as ammonia.

In addition to amino-sugar(s) there must therefore be present in the polysaccharide a nitrogenous compound which yields its nitrogen partly as amino-nitrogen and partly as ammonia on acid or alkaline hydrolysis, the proportion of ammonia being probably larger in the latter case (see next paragraph). This compound has not so far been identified; colour reactions and consideration of its properties seem to exclude all known amino-acids and its instability both towards acid and alkali make its closer investigation difficult. It is hoped that

its further study may become possible if success is attained in the search for an enzyme which will hydrolyse the polysaccharide.

Action of alkali. Fig. 2 shows that 50 % of the total nitrogen is liberated from the polysaccharide as ammonia by N NaOH at 100° after 16 hr. The fact that the yield of mannose as phenylhydrazone after a subsequent acid hydrolysis was greatly diminished indicated that the sugar had been partially destroyed by the alkaline treatment. Similarly, the fact that only 60 % of the remaining nitrogen or 30 % of the original nitrogen was present as amino-nitrogen showed that some
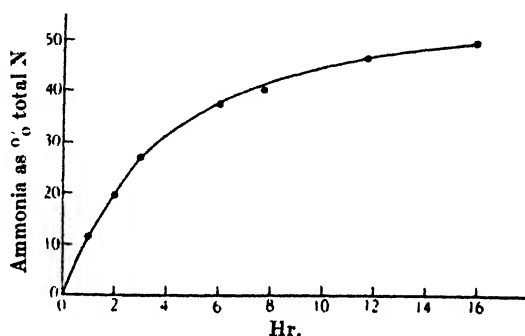
Fig. 2. Rate of liberation of ammonia on hydrolysis of polysaccharide with N NaOH at 100°.

glucosamine also had been destroyed. This apparent instability of glucosamine bound in a polysaccharide towards strong alkali is in contrast to the behaviour of methyl glucosaminide which is very stable under the conditions used [Moggridge & Neuberger, 1938].

After alkaline hydrolysis the polysaccharide gives a positive test with Ehrlich's reagent which is not easy to explain, since N-acetyl methyl glucosaminide and N-acetyl phenyl glucosaminide, when heated with alkali under the same conditions, do not give this reaction. A possible explanation is that a part at least of the glucosamine is bound to the unknown nitrogenous substance by a linkage more unstable to alkali than the O-glucoside linkage. On alkaline treatment, this linkage might be split more quickly than the N-acyl linkage, and the compound thus partly deaminated and partly condensed to a ring system giving a positive test with Ehrlich's reagent.

Acetyl content. Acetyl estimations showed that the polysaccharide contained 10·65 % acetyl groups, presumably in the form of N-acetyl. The amount of acetyl corresponds nearly exactly to the amount of amino-nitrogen obtained after acid hydrolysis, and it may be concluded that the acetyl groups are linked partly to glucosamine and partly to the unknown nitrogenous component. Since the isolation of the polysaccharide involves acetylation it is impossible to say whether the glucosamine in its original combination in the protein is N-acylated or not.

Content of the polysaccharide in egg albumin. As will be shown later, 3·5 g. of the polysaccharide complex are obtained from 100 g. of egg albumin. If the yield of the polysaccharide is assumed to be 95 %, this corresponds to a value of 3·65 g. per 100 g. of protein. Since the mannose content of the polysaccharide complex is 50 %, a value of 1·8 g. of mannose per 100 g. of protein is obtained which is in perfect agreement with the colorimetric value. From the analytical figures therefore egg albumin with mol. wt. 40,000 would contain 4 mol. of mannose and 2 mol. of glucosamine.

Estimations of the mol. wt. of the polysaccharide complex indicated a value of 1250, which, on the basis of mol. wt. 40,000 for egg albumin, corresponds to a polysaccharide content of the protein of 3·15%. The agreement with the value obtained from isolation and colorimetric methods is sufficiently good to justify the assumption that four mannose and two glucosamine residues are linked up as one polysaccharide chain with the protein to form one large prosthetic group.

EXPERIMENTAL

Preparation of egg albumin

Egg albumin was crystallized according to Kekwick & Cannan [1936]. Samples from each crystallization were dialysed against frequent changes of distilled water for 10 days and nitrogen and carbohydrate were estimated. For the estimation of carbohydrate the method of Sørensen & Haugaard [1933] was used employing mannose as standard. The nitrogen content of egg albumin was assumed to be 15·65%.

Effect of heat-denaturation on carbohydrate content

Five times recrystallized egg albumin (20 g.) was denatured by heating in aqueous solution at 80° for 20 min. and the suspension was dialysed against frequent changes of distilled water at 0°. The combined dialysates were evaporated to dryness in vacuo. A minute amount of solid matter giving a very weak Molisch reaction was obtained. It was impossible to obtain from it an osazone, even after acid hydrolysis, nor could a phenylhydrazone or phenylhydrazide be prepared; the presence of hexoses and hexonic acids was thus excluded.

Ultrafiltration experiments

The membranes were made and standardized by Dr Elford and the technique used was that described by Elford & Ferry [1934]. Nitrogen and carbohydrate were estimated in samples of the supernatant fluid and of the ultrafiltrate. Heat-denatured protein was prepared by keeping an electrolyte-free solution of the protein at 85° for 20 min. The suspension was centrifuged at 2000 r.p.m. for 5 min. and the supernatant fluid was filtered through a 1·5μ membrane which allowed about 12% of the protein originally present to pass through. Acid-denatured protein was prepared by keeping a protein solution for 10 hr. at pH 1 at a temperature of 30° and then adjusting the pH to the isoelectric point. Protein thus denatured passes readily through a 1·5μ membrane, but is completely retained by a 15mμ membrane.

Isolation of the polysaccharide from egg albumin

Egg albumin, five times recrystallized and nearly completely freed from sodium sulphate by dialysis, was used in all experiments. A 5% solution of the protein was denatured by heating and the reaction of the suspension was adjusted to pH 7·8. Trypsin (Merck) was then added in an amount corresponding to 0·3% of the dry weight of the protein. Alkali was added frequently to adjust the pH of the digest to 7·8 and further quantities of trypsin were added about twice a week. The enzymic hydrolysis was followed by alkalimetric titration in 90% alcohol according to Willstätter & Waldschmidt-Leitz [1921], correction being made for the alkali added; the digestion was continued for 4–6 weeks. The total amount of trypsin added varied between 0·8 and 1·1 g. per 100 g. of protein.

The working up of this digestion mixture may be described in detail for a typical experiment.

The enzymic hydrolysate of 120 g. of egg albumin was acidified to pH 1 with HCl. Phosphotungstic acid (150 g.) in $0.1 N$ HCl was added and the mixture was left for 3 days in the cold room. The precipitate was filtered off and washed with a 10 % solution of phosphotungstic acid in $0.1 N$ HCl. The precipitate was suspended in warm 50 % acetone in which most of it dissolved and the suspension was extracted five times with an amyl alcohol-ether mixture. The aqueous suspension was filtered and the solution was analysed for carbohydrate and nitrogen (fraction I). The precipitate was again suspended in water and dissolved by addition of NaOH (fraction II). The phosphotungstic acid was removed by $Ba(OH)_2$ and the excess $Ba(OH)_2$ precipitated by H_2SO_4.

The filtrate from the phosphotungstic acid precipitation was freed from excess phosphotungstic acid by amyl alcohol-ether extraction until a sample gave no precipitation with $Ba(OH)_2$. The solution was then neutralized with $2 N$ NaOH and concentrated to low bulk.

To the neutralized solution sufficient NaOH was then added to adjust the reaction to pH 9 and ketene was passed in until the ninhydrin reaction became negative; this took about 18 hr. Small additions of alkali were made at intervals to maintain the pH at about 6. The solution was then concentrated at low pressure and acidified to pH 1·6 with HCl. Acetylated amino-acids were now removed by continuous extraction with chloroform in the apparatus shown in Fig. 3. The extraction was generally stopped after 12 hr. since it was found that continuance for a further 50 hr. did not appreciably increase the amount of nitrogen in the chloroform layer. The chloroform solution was evaporated to dryness, the residue was taken up in water and nitrogen and carbohydrate were estimated.

The aqueous solution was concentrated *in vacuo* and dried in a desiccator first over conc. H_2SO_4 and finally over P_2O_5 to a thick syrup. To this syrup were added pyridine (250 ml.) and acetic anhydride (350 ml.), and the mixture was shaken mechanically for 2 days at 2°. It was then poured into ice-water and left for 5 hr. The solution was concentrated under very low pressure, the residue diluted with water and the latter again evaporated. The aqueous solution of the residue was then made acid to Congo red and extracted repeatedly with chloroform, the combined chloroform extracts being washed with water containing NaCl and dried. After removal of the chloroform an oil was obtained which was dissolved in alcohol. Sufficient lithium hydroxide was added to keep the solution

Fig. 3.[1] Apparatus for continuous extraction of an aqueous solution with chloroform.

alkaline to phenolphthalein for at least half an hour. After 2 hr. more in the ice-chest the alkali was exactly neutralized with the required amount of dilute HCl and the solution concentrated under reduced pressure nearly to dryness; the residue was dissolved in a small amount of water and alcohol was added to a final concentration of 95–97 %. The precipitate was centrifuged off

[1] This apparatus can be purchased from Messrs Quickfit and Quartz, Ltd.

after standing for several hours in the ice-chest, washed several times with ice-cold absolute alcohol and dried over P_2O_5.

The mother liquor from the alcohol precipitation was evaporated and a further small amount of precipitate was obtained on addition of alcohol. Altogether 2·9 g. of material were obtained.

The aqueous solution obtained from the last chloroform extraction and containing unacetylated polysaccharide was evaporated to dryness, and subjected to reacetylation followed by further treatment as above; 1·2 g. more of material were obtained, identical in all respects with that derived from the first acetylation. A third acetylation gave no further product.

General properties of the polysaccharide

The substance obtained by the method described is very slightly pigmented and fairly hygroscopic; it is very soluble in water, somewhat soluble in aqueous alcohol and insoluble in absolute alcohol and acetone. Its aqueous solution has pH 5·5–6·0. The nitrogen contents of different samples as estimated by the Kjeldahl method varied between 4·85 and 5·1%. (It was found that reliable nitrogen values for glucosamine or glucosamine-containing compounds could only be obtained by the ordinary Kjeldahl method if ashing with H_2SO_4 were continued for about 6 hr.; with SeO_2 as catalyst however half an hour sufficed.)

The polysaccharide gives no biuret reaction even in a 20% solution; it gives a ninhydrin reaction only after acid hydrolysis. The Millon and Sakaguchi reactions are negative both with the untreated polysaccharide and with samples hydrolysed with $2N$ HCl and with conc. HCl. In view of the claim of Rimington [1931] that histidine is combined with the polysaccharide obtained from serum proteins the Pauly reaction was carried out on samples hydrolysed under many different conditions: 5 mg. samples were hydrolysed with $2N$ HCl at $100°$, conc. HCl at $125°$ and with 10% $Ba(OH)_2$ at $100°$ all for 5 hr. In all cases only a slight yellow-orange colour was obtained with diazobenzenesulphonic acid in sodium carbonate solution, which was very different from the colours given by histidine and tyrosine. The Hopkins-Cole and Rosenheim tests for tryptophan were negative before and after alkaline hydrolysis. Tests for sulphur and phosphorus were likewise negative.

The Molisch reaction was positive up to a dilution of 1 : 1,000,000. The polysaccharide itself did not reduce Fehling's solution, but became strongly reducing on mild acid hydrolysis. A strongly positive test for N-acetylglucosamine [Morgan & Elson, 1934] was obtained after hydrolysis with 0·01N HCl at $100°$ for 1 hr. The naphthoresorcinol, phloroglucinol and Seliwanoff tests were all negative on samples hydrolysed under difficult conditions. Carbohydrate estimations by the method of Sørensen & Haugaard gave values of 47–50% using mannose as a standard (see Table IV).

Estimation of the molecular weight

The molecular weight was estimated by an isopiestic method worked out by Dr G. S. Hartley similar to that described by Robinson & Sinclair [1934]. A rectangular silver plate containing three circular grooves was placed in a bulb-shaped glass apparatus which could be exhausted. Polysaccharide solutions of different concentrations were placed in the outside grooves while a solution of KCl of known strength was put into the middle groove. The apparatus was carefully exhausted on an oil pump and left for 2 days at $25°$; it was then opened and the amounts of solutions in the three grooves were determined by weighing.

Osmotic equilibrium was reached, and the concentrations of polysaccharide in the two outside grooves were identical.

The method may be described in detail for one experiment. 0·0896 g. of a solution containing 0·00706 g. of polysaccharide was placed in groove 1, 0·05840 g. of a solution containing 0·00132 of polysaccharide in groove 3, whilst in groove 2 0·09168 g. of 0·05 N KCl was introduced. At the end of the experiment grooves 1 and 3 contained 0·0928 and 0·0178 g. of solution respectively indicating concentrations of polysaccharide of 7·45 and 7·61 %. The values show that osmotic equilibrium between the two polysaccharide solutions had in fact been established, and it can be assumed that equilibrium had also been reached between the polysaccharide and the KCl solutions. In groove 3 containing KCl, the amount of solution had increased to 0·117 g. indicating that the two polysaccharide solutions are in equilibrium with a 0·0409 N solution of KCl. Using an osmotic coefficient for KCl of 0·943 and recalculating the concentrations of polysaccharide in g. per 1000 g. of solvent, values for the mol. wt. of 1120 and 1150 respectively were obtained.

Estimation of ash content

10·62 mg. of the polysaccharide were ashed by heating at 500° for 6 hr. The residue amounted to 0·0192 mg. corresponding to an ash content of 0·1735 %. If it is assumed that the ash consists only of lithium chloride a correction for the value of the mol. wt. can be made. The values of the mol. wt. corrected on such a basis are 1245 and 1275.

The homogeneity of the polysaccharide

450 mg. of the polysaccharide were dissolved in 1 ml. of water and 1 ml. of alcohol was added. The precipitate was centrifuged off and dried. To the supernatant solution pure alcohol was added and the precipitate formed again centrifuged off. By thus increasing the alcohol concentration several fractions were obtained. Optical rotations, mol. wt. and nitrogen and carbohydrate contents of the different fractions were estimated; the results are recorded in Table IV.

Estimation of reducing sugars

66·6 mg. of the polysaccharide were dissolved in 11 ml. of 1·5 N HCl and heated at 100° using a reflux condenser. 1 ml. samples were taken out at intervals, neutralized to 99 % with NaOH and made up to 2·5 ml. The reducing values were estimated according to Hanes's [1929] modification of the Hagedorn-Jensen method. The results in terms of glucose are recorded in Fig. 1. The reducing value rises rapidly on hydrolysis until it reaches a maximum at about 6 hr.; thereafter it decreases slowly, presumably owing to destruction of the sugar. The maximum reducing value expressed as glucose corresponds to a content of 85 % of reducing sugar in the polysaccharide. This value is too high for two reasons. Mannose and glucosamine which occur in the polysaccharide have higher reducing equivalents than glucose: expressed in terms of glucose the molecular reducing equivalents of mannose and glucosamine were found by the Hagedorn-Jensen method to be 101 and 103·5 % respectively. Secondly, it was noted that if an amount of sodium chloride is added to a glucosamine solution similar to that formed on neutralizing the acid hydrolysate, the apparent reducing value of glucosamine is increased by about 10–12 % (see Moggridge & Neuberger, [1938]). If correction was made for these two factors a value of about 75 % of reducing sugar in the polysaccharide was obtained.

Estimation of fermentable sugar

30·2 mg. of polysaccharide were dissolved in 6 ml. of 1·5N HCl and heated in a sealed tube at 100° for 6 hr. The solution was then neutralized and made up to 20 ml. One 2 ml. sample of this solution was incubated with washed baker's yeast at 37° for 20 min. according to the method of Somogyi [1929]. The yeast was centrifuged off and the reducing value of the supernatant solution estimated. In a second sample the reducing value was estimated without previous treatment with yeast. Controls with yeast alone were carried out at the same time. The amount of sugar expressed as glucose in the sample not treated with yeast was 2·5 mg., whilst in the sample treated with yeast it amounted only to 0·81 mg. after correction for the reducing value of yeast itself had been made.

Estimation of amino-nitrogen

The polysaccharide itself does not give off any nitrogen in the Van Slyke apparatus. On hydrolysis the following amounts of amino-nitrogen are found:

	%
After 5 hr. hydrolysis with 1·5N HCl	3·2
After 8 hr. hydrolysis with 2·5N HCl	3·6
After 8 hr. hydrolysis with 5N HCl	3·2

These values were obtained on shaking for 5 min. In one experiment—after hydrolysis with 5N HCl—shaking was continued for 30 min. and the value was increased to 4·2%. The apparent increase on prolonged shaking is probably due to the ammonia formed on acid hydrolysis. It may be noted that glucosamine hydrochloride gives off all its nitrogen on shaking for 5 min.

Acetyl estimation

Acetyl groups were determined according to Pregl & Soltys [1929]. In two experiments values of 10·61 and 10·67% of acetyl were obtained.

Estimation of amino-sugar

Amino-sugar was estimated by Sørensen's [1938] modification of the method of Elson & Morgan [1933]. A standardization curve was obtained with solutions of glucosamine hydrochloride; the colorimetric readings were done on a Zeiss step-photometer. The mean value from several experiments corresponded to a content of 29·1% of amino-sugar expressed as glucosamine hydrochloride or 24·3% expressed as glucosamine.

Isolation of mannose

(a) *Isolation as phenylhydrazone.* 99 mg. of polysaccharide were dissolved in 1 ml. 1·5N HCl and heated in a sealed tube at 100° for 6 hr. The neutralized solution was filtered and made up to 3 ml. To this solution 0·2 ml. of glacial acetic acid and 0·3 ml. of freshly distilled phenylhydrazine were added. After a short time a crystalline precipitate formed which was filtered off after standing for 4 hr., washed with two lots of 0·5 ml. of 15% acetic acid and dried. It amounted to 51 mg. and had M.P. after two recrystallizations 189° (uncorr.); a sample mixed with authentic mannose phenylhydrazone showed no depression of M.P. (Found: N (Dumas), 10·1%. $C_{12}H_{18}O_5N_2$ requires N, 10·33%.)

(b) *Isolation as* p-bromophenylhydrazone. 100 mg. of polysaccharide were dissolved in 10 ml. 1·5N HCl and heated at 100° for 5½ hr. The solution was evaporated to dryness at reduced pressure, the temperature of the bath not

exceeding 36°. The residue was taken up in water, neutralized and treated with 150 mg. *p*-bromophenylhydrazine in 50 % acetic acid. Crystallization started at once; after 4 hr. standing the precipitate was filtered off, washed with 25 % alcohol and dried. It amounted to 71 mg.

The twice recrystallized hydrazone had M.P. 202° (uncorr.); a sample mixed with authentic mannose *p*-bromophenylhydrazone showed no depression of M.P. No osazone could be obtained from the residual solution.

Isolation of glucosamine

Difficulties were encountered in attempts to separate glucosamine from hydrolysates of the polysaccharide until a new derivative of glucosamine was found which is very suitable for purposes of isolation. Glucosamine condenses at slightly alkaline reaction with 2:4-dihydroxybenzaldehyde to form a Schiff's base which crystallizes readily and which, in contrast with other Schiff's bases of glucosamine, is stable towards excess alkali. The reaction is almost quantitative as shown by the following experiments.

Preparation of 2:4-dihydroxybenzylidene-1-glucosamine. 215 mg. glucosamine hydrochloride (1 mol.) and 150 mg. $KHCO_3$ (1·5 mol.) were dissolved in 2·5 ml. water. 175 mg. 2:4-dihydroxybenzaldehyde (1·25 mol.) dissolved in 2 ml. 40 % ethyl alcohol were added to the solution. Crystallization started after 15 min.; the mixture was left overnight in the ice-chest and the yellow crystals were then filtered off and washed with 2 ml. 40 % alcohol. Yield: 265 mg. or 90 % of the theoretical. The yield is not decreased by the presence of other sugars.

The substance was recrystallized from aqueous alcohol. It is soluble in alcohol and hot acetone, insoluble in water and ether. The substance has M.P. 116° (uncorr.) and decomposes at 118°. (Found: N (Kjeldahl), 4·54 %. $C_{13}H_{17}O_7N$ requires N, 4·67 %.) The substance mutarotates; the initial $[\alpha]_D = 312°$ (in alcohol); after 42 hr. $[\alpha]_D = 228°$.

If the substance is recrystallized by adding a large amount of water to its concentrated alcoholic solution and leaving the mixture overnight a new compound is formed which is pale yellow in colour and has M.P. 142°. Analysis indicates that the elements of water have been added to the Schiff's base. The same change occurs if the non-hydrated form is kept for some time exposed to the air. (Found: C, 49·15; H, 6·05 %. $C_{13}H_{17}O_7N$ requires C, 49·27; H, 6·04 %.)

The rotation of the new compound is slightly different from that of the Schiff's base; $[\alpha]_D$ (initial) = 299° (in alcohol), after 42 hr. $[\alpha]_D = 198°$.

Formation of a hydrochloride. 2:4-Dihydroxybenzylideneglucosamine forms with hydrochloric acid a hydrochloride of the hydrated compound containing 1 equiv. HCl. 280 mg. of the Schiff's base were dissolved in hot acetone and 0·25 ml. 5N HCl was added. A white crystalline precipitate formed at once which was filtered off and dried. It amounted to 320 mg. The substance is easily soluble in water and slightly soluble in alcohol, insoluble in ether and acetone. (Found: N, 3·8; Cl, 9·8 %. $C_{13}H_{20}O_8NCl$ requires N, 3·96; Cl, 10·04 %.)

An alcoholic solution treated with an equivalent amount of aqueous acid gives an identical product.

As found by Dimroth [1902] 2:4-dihydroxybenzylideneaniline is able to add on the elements of water and to form a hydrochloride. By analogy with his interpretation, the reactions here described may be represented as follows:

$$C_6H_{11}O_5 . NH_2 + HOC . C_6H_5O_2 \rightarrow C_6H_{11}O_5 . N{:}CH . C_6H_5O_2 + H_2O$$
$$+ HCl$$
$$\rightarrow C_6H_{11}O_5 . NH . CH(OH) . C_6H_5O_2 \rightarrow C_6H_{11}O_5 . NH_2^+ . CH(OH) . C_6H_5O_2 + Cl^-.$$
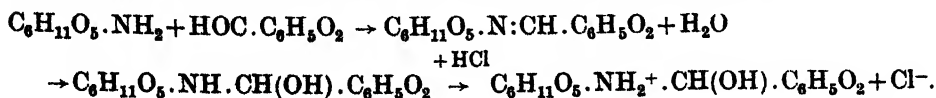
Recovery of glucosamine. Although the 2:4-dihydroxybenzylidene compound is quite stable to acid in the cold, it is split quantitatively on heating in acid solution.

150 mg. of dihydroxybenzylidene glucosamine were dissolved in 5 ml. 65% alcohol containing 1·5 N HCl and heated under reflux on a water bath for 30 min. The crystalline precipitate which formed on cooling was increased by the addition of ether. The precipitate was filtered off and dried. The product was identified by nitrogen content and rotation as glucosamine hydrochloride, and the yield was 90% of the theoretical.

Isolation of glucosamine from the polysaccharide. 500 mg. of polysaccharide were hydrolysed with 5 ml. 5 N HCl for 3 hr. at 100°. The solution was filtered and evaporated nearly to dryness under reduced pressure; the residue was taken up in water and the solution was again filtered. 500 mg. of phosphotungstic acid dissolved in 2·5 ml. 0·1 N HCl were now added and the mixture was left overnight. The precipitate was centrifuged off and washed twice with, altogether, 2·5 ml. of a 5% phosphotungstic acid solution in 0·1 N HCl. The combined solution and washings were extracted with amyl alcohol-ether and the aqueous solution was concentrated to low bulk, again filtered and allowed to evaporate to dryness in a desiccator. The residue which consisted of large plates admixed with amorphous material, was taken up in a small quantity of water, heated with alcohol and again evaporated to remove the last traces of water. Absolute alcohol was now added and the crystalline material was filtered off. It amounted to 120 mg.

The crystalline material was dissolved in 3 ml. 30% alcohol to which $KHCO_3$ (80 mg.) and 2:4-dihydroxybenzaldehyde (100 mg.) dissolved in 0·5 ml. 30% alcohol were added. After 6 hr. in the ice-chest the crystals were filtered off and amounted to 80 mg. The material was dissolved in alcohol and filtered; the solution was evaporated nearly to dryness and the residue recrystallized by addition of water. The substance had M.P. 142°; a sample mixed with the hydrated form of 2:4-dihydroxybenzylideneglucosamine showed M.P. 141°. It agreed with the latter also in optical rotation, having $[\alpha]_D$ (initially) $= 304°$ (in alcohol), after 39 hr. $[\alpha]_D = 204°$. (Found: C, 49·20; H, 6·01%. $C_{13}H_{19}O_6N$ requires C, 49·27; H, 6·04%.)

Isolation of glucosamine hydrochloride. 35 mg. of the Schiff's base were dissolved in 2 ml. 1·5 N HCl in 65% alcohol and the solution heated for 30 min.; the crystalline material was filtered off, washed with anhydrous ether and dried. Yield: 22·8 mg. (Found: C, 32·75; H, 6·51; N, 6·38%. $C_6H_{14}O_5NCl$ requires C, 33·4; H, 6·54; N, 6·50%.) In experiments involving different conditions of hydrolysis no better yields of the dihydroxybenzylidene compound could be obtained.

Nitrogen precipitated by phosphotungstic acid after acid hydrolysis

The phosphotungstic acid precipitate was decomposed with hydrochloric acid and amyl alcohol-ether and the aqueous solution was made up to known volume. A nitrogen estimation showed that 28% of the total nitrogen of the polysaccharide was precipitable by phosphotungstic acid after acid hydrolysis. The polysaccharide itself is not precipitated by phosphotungstic acid. The Pauly reaction was negative and no reaction for pyridine (chlorodinitrobenzene) could be obtained in the solution recovered from the phosphotungstate. Ammonia estimations by the method of Conway & Byrne [1933] indicated that about 55% of the nitrogen contained in the precipitate consisted of ammonia. (The ammonia was identified by analysis in the form of ammonium chloride.) Colorimetric

tests with Nessler's reagent indicated that the ammonia is not formed by the alkali used in its estimation. The residual nitrogen contained in the precipitate could not be accounted for; all attempts to prepare derivatives (chloroplatinate, chloroaurate, picrate, picrolonate, reineckate) led only to amorphous gums.

If milder conditions were used for acid hydrolysis the amount of ammonia present in the phosphotungstic acid precipitation was smaller, but it was still impossible to obtain crystalline products from this fraction.

Alkaline hydrolysis

Only small amounts of volatile base are evolved on boiling the polysaccharide with dilute alkali; e.g. 1·4 or 2·8% of the total nitrogen were obtained as volatile base on boiling the polysaccharide for 1 hr. with 0·01N and 0·1N NaOH respectively, but much larger amounts are obtained on boiling with N NaOH.

51·1 mg. of polysaccharide were dissolved in 8 ml. N NaOH and the solution was boiled under reflux, the vapours being trapped in a receiver containing a known amount of 0·01N HCl. The acid solution was back-titrated against 0·01N NaOH at suitable intervals, and the results are recorded in Fig. 2. The experiment was stopped after 16 hr. In another experiment with 500 mg. polysaccharide the combined solutions containing the volatile base were made alkaline and distilled in the micro-Kjeldahl apparatus into a receiver containing HCl. The distillate was evaporated to dryness and the crystalline residue dried. The total yield was 48 mg. 10·3 mg. were used for nitrogen estimation. (Found: N, 26·05%. NH_4Cl requires N, 26·16%.)

The alkaline solution of the polysaccharide was acidified and hydrolysed for 5 hr. with 1·5N HCl. Only about 100 mg. of mannose phenylhydrazone could be obtained, a yield of 14% as against 37% obtained from non-alkali-treated polysaccharide.

In another experiment in which 500 mg. of the polysaccharide were hydrolysed with 10% $Ba(OH)_2$ at 100° for 7 hr. 37% of the total nitrogen was given off as ammonia. Ba^{++} was removed exactly from the alkaline solution by H_2SO_4 and $BaSO_4$ was filtered off. The solution of the polysaccharide after alkaline hydrolysis gave a strong reaction with Ehrlich's reagent. To the solution were now added 5 ml. of Hopkins's reagent ($HgSO_4$ in 5% H_2SO_4) and the mixture was left for 2 days. The mercuric sulphate precipitate was decomposed by H_2S and $Ba(OH)_2$ and the solution was evaporated nearly to dryness under reduced pressure. No colour reaction for tryptophan could be obtained nor was it possible to prepare a crystalline derivative from this fraction.

DISCUSSION

It is felt that the work described in this paper places beyond all doubt the question of the occurrence of carbohydrate in crystalline egg albumin. The constancy of carbohydrate content on repeated crystallization and the consistent failure of all attempts at physical separation of protein and carbohydrate fractions, even by processes such as denaturation which must involve intra-molecular change, leave no escape from the conclusion that a constant amount of carbohydrate is firmly attached to the protein molecule.

The second conclusion which emerges from the present work concerns the nature of the carbohydrate group, since for the first time a method has been devised for the isolation of this group in a condition which may be assumed to be essentially unaltered. Viewing the properties of the complex isolated from egg albumin in the light of this assumption we find that it consists of

a polysaccharide of molecular weight about 1200, made up for the most part of mannose and glucosamine in the proportion of two molecules of the former to one of the latter, but containing also an unidentified nitrogenous constituent. In all probability the glucosamine occurs in the actual protein-carbohydrate complex in the acetylated condition as it does in other natural products containing glucosamine, such as chitin, but this point cannot be decided by the method at present available; nor can the possibility of the presence of an amino-sugar other than glucosamine in the egg albumin polysaccharide be completely excluded, although it seems extremely remote.

Unfortunately the methods employed in the present work are incapable of giving us any definite information concerning the mode of combination of the polysaccharide with the rest of the protein molecule, except in so far as the liberation of the polysaccharide by tryptic hydrolysis suggests the participation of a peptide linkage; it is possible that the unidentified nitrogenous constituent of the polysaccharide may play some part in the combination and that a knowledge of the nature of this constituent might enable us to assign a definite role to the polysaccharide in the structure of the natural protein. A more exact analysis of the situation must await the development of new methods of attack and further speculation at the present time serves no useful purpose; it is even now however possible to state with some certainty, on the basis of the findings recorded above, that the polysaccharide forms a single prosthetic group with one point of attachment to the molecule of egg albumin, the word prosthetic implying no more than that the group in question is made up of compounds other than amino-acids.

One more point arises which may be briefly considered here. The discovery in recent years of the predominant part which is played by carbohydrate groups in the determination of the specificity (as distinct from antigenic capacity) of many bacterial antigens naturally gives rise to the speculation that a similar state of affairs may exist among the proteins in so far as the latter contain carbohydrate groups. Hitherto it has been impossible to put this question to the test since no method has been available whereby the carbohydrate group could be separated from a protein without subjection to treatment calculated to alter its biological properties entirely. With the aid of the gentle method of isolation described in the present paper it seems that the problem can be tackled with more hope of success, and experiments are in fact already in hand to examine how far, if at all, the immunological specificity of crystalline egg albumin is determined by the carbohydrate group which it contains.

SUMMARY

1. Previous work on the occurrence of carbohydrate in proteins other than mucins and mucoids is briefly reviewed.

2. It is shown that the carbohydrate content of crystalline egg albumin becomes constant on recrystallization and that it is impossible to separate the carbohydrate from the protein by denaturation or ultrafiltration.

3. A method for the isolation of the carbohydrate group in almost quantitative yield from egg albumin is described which is based on tryptic hydrolysis and involves no chemical treatment more drastic than acetylation and deacetylation under the mildest conditions.

4. The properties of the polysaccharide thus isolated are described in detail. It is shown to have mol. wt. about 1200 and to be composed almost certainly of four molecules of mannose and two of glucosamine, together with an unidentified nitrogenous constituent. The complex is regarded as forming a single

prosthetic group with one point of attachment to the molecule of natural egg albumin.

5. The preparation and properties are described of 2:4-dihydroxybenzylideneglucosamine, a compound useful for the isolation and characterization of glucosamine.

6. The possible significance of the carbohydrate group in relation to the structure and immunological properties of egg albumin is briefly discussed.

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CXC. NOTE ON THE ACTION OF ACETYLATING AGENTS ON AMINO-ACIDS

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IN connexion with the development of a new method for the isolation of carbohydrate from protein hydrolysates [Neuberger, 1938] it became necessary to study certain aspects of the reactions of amino-acids and related compounds with pyridine-acetic anhydride and ketene.

Reactions of amino-acids with pyridine and acetic anhydride

If amino-acids are treated in glacial acetic acid solution with the theoretical amount of acetic anhydride, optically active acetamino-acids are obtained, while if a large excess of acetic anhydride is used, racemization and/or formation of azlactones takes place [Bergmann *et al.* 1926; Bergmann & Zervas, 1928]. Excess of acetic anhydride causes racemization even in aqueous solution in the presence of alkali [du Vigneaud & Meyer, 1932]. If the reaction with acetic anhydride, however, is carried out at 100° in the presence of pyridine, CO_2 is split off and acetaminoketones result [Dakin & West, 1928]. It has been found by the present author that this anomalous reaction is suppressed at low temperature. Thus two typical amino-acids, histidine and phenylalanine, which by treatment with pyridine and acetic anhydride at 100° are almost quantitatively converted into the corresponding acetaminoketones, give, when the reaction is carried out at 2°, very good yields of acetamino-acids; only in one case was there an indication that a minute trace of a ketone was formed. It seems, however, that at least partial racemization of some amino-acids is induced by this method of acetylation also.

Action of ketene on amino-acids and related compounds

Bergmann & Stern [1930] found that the alkali salts of simple amino-acids such as leucine and phenylalanine, or in some cases the amino-acids themselves, react with ketene to form *N*-acetylated compounds whilst tyrosine in presence of an excess of alkali gives *ON*-diacetyltyrosine. This method of acetylation which is quantitative and does not lead to side-reactions has been used to mask amino- and phenolic hydroxyl-groups in proteins [Herriott & Northrop, 1934; Stern & White, 1938] and it has been tacitly assumed that changes in the protein molecule, under the action of ketene, are in fact restricted to these two types of groups. The experiments of Bergmann & Stern [1930] were confined to racemic monoaminomonocarboxylic acids, *l*(−)-tyrosine and *l*(+)-glutamic acid and the question of racemization was not studied in detail by these authors; in the present work the study of the reaction has been extended to more complex amino-acids, in which cases, as will appear, partial or complete racemization may occur.

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As might be expected, the α-imino-group of proline is readily acetylated by ketene in alkaline solution. Basic amino-acids on the other hand react only with their amino-groups: thus from *l*(+)-arginine and *l*(−)-histidine the corresponding mono-acetyl compounds were obtained. Lysine, however, reacts with ketene to form a diacetyl derivative. It may be noted in passing that although the glyoxaline group of histidine does not react with ketene in aqueous solution, glyoxaline and 4-(or 5)-methylglyoxaline when dissolved in indifferent solvents are changed by ketene into non-crystalline unsaturated compounds not giving the Pauly reaction. Probably ring fission and simultaneous acetylation occur and the acetylated unsaturated diamines [Bamberger & Berlé, 1893] formed in the first instance, undergo polymerization.

Aliphatic hydroxyl groups do not react readily with ketene even in indifferent solvents as shown by the fact that from ethyl *dl*-β-hydroxyglutamate in ether the *N*-acetyl compound only was obtained. In marked contrast to this is the behaviour of aliphatic thiol groups. If cysteine dissolved in 2 equiv. of alkali is treated with ketene both the amino- and thiol-groups are acetylated; α-thiolpropionic acid is likewise acetylated in aqueous alkali.

However, if α-thiolpropionic acid is treated with ketene in ether instead of water, a mixed anhydride of S-acetyl-α-thiolpropionic and acetic acids is formed. The mixed anhydride which can be distilled decomposes in the presence of water to form S-acetyl-α-thiolpropionic acid; this reaction is not surprising since ketene is known to form mixed anhydrides with carboxylic acids in indifferent solvents [Hurd & Dull, 1932]. Dicarboxylic acids, however, react with ketene in indifferent solvents to form internal anhydrides; thus from β-phenyl-glutaric acid the corresponding anhydride was readily produced.

It was noticed in several cases, especially when an excess of alkali was used, that the action of ketene on amino-acids resulted in partial or complete racemization. This is probably due to the action of acetic anhydride, which is almost certainly the first reaction product between ketene and water, and which, as mentioned above, racemizes acetamino-acids at alkaline reactions. Thus arginine is completely racemized, in agreement with the observation of du Vigneaud & Meyer [1932] who found that *N*-acetyl-*l*(+)-arginine is racemized by acetic anhydride even without addition of alkali.

From these results it can be concluded that if proteins are treated with ketene in alkaline solution acetylation may take place at amino-, thiol- and phenolic-groups, whilst guanidino- and aliphatic hydroxyl-groups will remain unaffected. The possibility that the glyoxaline group might react with ketene, when the carboxyl group is blocked, cannot be completely excluded: it is well known that the Bamberger fission does not take place with substituted glyoxalines containing free carboxyl groups in the side chain whilst the esters of such compounds undergo the fission in the normal manner [Windaus *et al.* 1921]; it is therefore possible that, although the glyoxaline ring of free histidine is not attacked by ketene in aqueous solution, the amino-acid may be so attacked when it is in peptide linkage.

EXPERIMENTAL

Action of pyridine and acetic anhydride on amino-acids in the cold

Preparation of acetyl-dl-phenylalanine. *dl*-Phenylalanine (1 g.) was suspended in a mixture of pyridine (5 ml.) and acetic anhydride (8 ml.) and the suspension was shaken mechanically for 2 hr. at 2°. The clear solution was left for 2 days at this temperature and then poured into ice-water. After standing for 4 hr. the solution was evaporated to dryness under reduced pressure,

the evaporation being twice repeated after addition of small amounts of water. The residue was taken up in a small amount of ethyl acetate, the ethyl acetate solution was dried and the substance was crystallized by addition of light petroleum. Yield was 85 % of the theoretical. Recrystallization was effected from water. At no stage of the preparation could a positive nitroprusside reaction for ketones be obtained. M.P. 148° (uncorr.). (Found: N (Kjeldahl), 6·85 %. $C_{11}H_{13}O_3N$ requires N, 6·76 %.) Titration: 20·9 mg. neutralized 5·10 ml. 0·02 N NaOH (cresol red). $C_{11}H_{13}O_3$, titrating as a monocarboxylic acid, requires 5·05 ml.

Preparation of acetylhistidine. $l(-)$-Histidine (1 g.) was suspended in pyridine (5 ml.) and acetic anhydride (8 ml.) and the mixture was shaken at 2° for 2 hr. and then left for 2 days at this temperature. Since part of the solid still remained undissolved the suspension was shaken for 6 hr. more at room temperature. The solution, which now contained only a small amount of solid matter, was poured into ice-water. A very faint reaction with nitroprusside was obtained. Removal of pyridine and acetic acid was carried out as described above. The residue obtained after evaporation was placed in a dish in a desiccator over absolute alcohol at a pressure of 60 mm. After a few hours the product crystallized out and was filtered off and dried. Yield 80 % of the theoretical. Recrystallization was effected by addition of acetone to a concentrated aqueous solution. (Found: N (Kjeldahl using SeO$_2$ as catalyst), 19·35 %. $C_8H_{13}O_4N_3$ requires N, 19·53 %.) M.P. 155° (not sharp) $[\alpha]_D = +22·5°$. Acetyl-$l(-)$-histidine [Bergmann & Zervas, 1928] has M.P. 169° and $[\alpha]_D = +44·7°$. It is clear therefore that partial racemization has occurred during acetylation.

Action of ketene on amino-acids and related compounds

Ketene was generated from acetone as described by Herriott [1934] in an all-glass apparatus in order to avoid the formation of polymerization products.

Preparation of acetyl-$l(-)$-*proline.* $l(-)$-Proline (250 mg.) was disssolved in 25 ml. 0·4 N NaOH and treated with ketene for 45 min. 5 ml. 2 N HCl were then added and the solution was evaporated to dryness under reduced pressure. The residue was taken up in alcohol, the solution filtered and again evaporated. The residue was taken up in ethyl acetate and crystallized by addition of light petroleum and recrystallized from chloroform and light petroleum. Yield was 80 % of the theoretical. M.P. 115° (uncorr.). (Found: N, 8·84 %. $C_7H_{12}O_3N$ requires 8·86 %. Titration: 12·6 mg. neutralised 4·01 ml. 0·02 N NaOH. $C_7H_{12}O_3N$ requires 3·98 ml.) $[\alpha]_D = -104°$. N-acetyl-$l(-)$-proline as described by du Vigneaud & Meyer [1932] has M.P. 117° (uncorr.) and $[\alpha]_D = -106·7°$.

*Preparation of acetyl-*dl-*arginine.* 1 g. of crystalline arginine prepared [Bergmann & Zervas, 1926] from the hydrochloride of $[\alpha]_D = +17°$, was dissolved in 25 ml. CO$_2$-free water and ketene was passed into the solution for 2 hr. The solution was evaporated to dryness and the residue was dried over KOH in a desiccator. The syrup was dissolved in a small quantity of water and crystallized by addition of alcohol. The product had M.P. 253° (decomp.) and lost no water on drying over P$_2$O$_2$ at 60° at 1 mm. (Found: N, 26·03 %. $C_8H_{11}O_3N_4$ requires 25·93 %.) A 5 % solution was optically inactive.

An inactive preparation of the acetyl compound was also obtained when a solution of $l(+)$-arginine hydrochloride ($[\alpha]_D +17·0°$) containing 2 equiv. of NaOH was treated with ketene at 0°.

Preparation of acetylhistidine. $l(-)$-Histidine, crystallized according to Vickery & Leavenworth [1927], was treated with ketene and the resulting

solution was worked up as described for arginine. The product was only partly racemized. It had $[\alpha]_D = +28.5°$; pure acetyl $l(-)$-histidine has $[\alpha]_D = +40.5°$.

Action of ketene on lysine. No crystalline acetyl derivative was obtained from lysine, but on treating 1 g. of $l(+)$-lysine dihydrochloride dissolved in water containing 3 equiv. of alkali with ketene a product was obtained which was soluble in alcohol and ethyl acetate. A solution, after ketene treatment, containing 5·7 mg. of total nitrogen per ml. gave 0·31 mg. of amino-nitrogen, in the Van Slyke apparatus, after shaking for half an hour, indicating that almost quantitative acetylation of both amino-groups had taken place.

Action of ketene on glyoxalines. Ketene was passed into a solution of 1 g. glyoxaline in anhydrous ether (20 ml.) which was kept at $-15°$ to avoid formation of polymerization products of ketene. An amorphous product was obtained which was easily soluble in water, slightly soluble in alcohol and insoluble in ether and did not give a reaction with diazobenzenesulphonic acid in Na_2CO_3 even after acid hydrolysis. A chloroform solution of the substance took up bromine and decolorized alkaline permanganate. Boiling the product with methyl alcoholic HCl [Windaus *et al.* 1921] led to an amorphous product which did not take up bromine any more and from which only a partly crystalline *p*-nitrophenylosazone was obtained; the latter could not be further purified. The product obtained by the action of ketene took up hydrogen in the presence of Pd but only to the extent of 25 % of the amount calculated for an ethylenediamine.

Similar products were obtained by the action of ketene on 4(or 5)-methylglyoxaline.

Preparation of ethyl N-acetyl-dl-β-hydroxyglutamate. Ethyl *dl-β*-hydroxyglutamate hydrochloride (2·2 g.) was dissolved in a small quantity of water; to the chilled solution ether and solid K_2CO_3 were added until the aqueous layer formed a thick paste. The paste was extracted several times with ether and the combined extracts were dried first with K_2CO_3 and then with freshly prepared BaO. Into the ethereal solution ketene was passed at $-15°$ for 1 hr. After removing the ketene by a stream of nitrogen the ethereal solution was concentrated to a syrup, which crystallized after standing in a desiccator at 0° for 2 days. Recrystallization was effected from ether. Yield was 53 % of the theoretical. M.P. 46° (uncorr.). (Found: N, 5·29%. $C_{11}H_{19}O_6$ requires N, 5·32%.) Acetyl content: found 17·1%. $C_{11}H_{19}O_6$ requires N, 16·47%.

Reaction of ketene with thiol groups

Preparation of NS-diacetylcysteine. $l(+)$-Cysteine hydrochloride (1 g.) was dissolved in a small quantity of water containing 3 equiv. of NaOH; ketene was passed into the solution for 1 hr. The solution was then acidified with 3 equiv. of H_2SO_4 and extracted with ethyl acetate; the residue from the evaporation of the combined and dried extracts was dissolved in a small quantity of ethyl acetate and, on addition of light petroleum (B.P. 120-140°), a crystalline precipitate was obtained. Yield 50 % of the theoretical. M.P. 111-112° (uncorr.). (Found: N, 6·84%. $C_7H_{11}O_4NS$ requires 6·83%.) Titration: 20·3 mg. neutralized 4·75 ml. 0·02N NaOH. $C_7H_{11}O_4NS$, as a monobasic acid, requires 4·95 ml. Acetyl estimation: acetyl found 41·3%; calculated for 2 acetyl groups 41·95%.

The substance is soluble in water, alcohol and most organic solvents. The nitroprusside reaction develops very slowly but the colour remains constant for many hours indicating a slow splitting of the thioester linkage by the ammonia employed in the reaction.

Preparation of S-*acetyl-α-thiolpropionic acid.* To a solution of freshly distilled α-thiolpropionic acid (5 g.) in freshly boiled out water (50 ml.) 30 ml. of $5N$ NaOH were added with exclusion of air. Ketene was passed into this solution for 2 hr. at 0°. The bulk of the acetic acid was then removed by extraction with ether; the solution was acidified with 30 ml. of $5N$ HCl and again extracted with ether. After removal of the ether an oil was obtained which could be distilled under very low pressure. It had B.P. 132–133° at 1 mm.; it was soluble in water, alcohol, ether and chloroform. (Found: S (Carius), 21·95 %. $C_5H_8O_3S$ requires S, 21·62 %.) The oil titrates as a monobasic acid; 142·4 mg. neutralized 9·69 ml. $0·1N$ NaOH (cresol red); calc. for $C_5H_8O_3S$, 9·62 ml. Saponification: 10 ml. $0·1N$ NaOH were added to the neutralized solution, and the mixture heated at 70° for 3 hr.; 1 ml. $0·1N$ HCl was then added and the solution was boiled to remove CO_2 and titrated with $0·1N$ NaOH to pH 7·5. Total amount of alkali required 9·65 ml.; calculated for 1 mol. of acid liberated 9·62 ml.

Preparation of a mixed anhydride of S-*acetyl-α-thiolpropionic and acetic acids.* 5 g. of α-thiolpropionic acid (freshly distilled) were dissolved in absolute ether (25 ml.) and treated with ketene. The solution was then evaporated to dryness and the oily residue which had a very pungent odour was dried in a desiccator over KOH and conc. H_2SO_4. It was finally obtained as a water-clear mobile liquid by distillation at 0·25 mm. with a bath-temperature of 77–83°. Yield was 5·59 g. (Found: S, 17·1 %. $C_7H_{10}O_4S$ requires S, 16·84 %.) The oil is soluble in most organic solvents, and insoluble in water; on prolonged contact with water, however, it dissolves and loses its pungent odour.

Preparation of S-*acetyl-α-thiolpropionic acid from the mixed anhydride.* 2·5 g. of the mixed anhydride were shaken with 5 ml. of water for 5 hr. when the oil had completely dissolved; the solution was then evaporated to dryness and the residue was left in a desiccator over KOH. It was then distilled: B.P. 133°/1 mm. (Found: S, 22·05 %. $C_7H_8O_3S$ requires S, 21·62 %.) Titration: 123·2 mg. required 8·36 ml. $0·1N$ NaOH for neutralization to cresol red; calculated for $C_5H_8O_3S$ as a monobasic acid 8·37 ml.

Preparation of β-*phenylglutaric acid anhydride.* β-Phenylglutaric acid (2 g.) dissolved in absolute ether (25 ml.) was treated with ketene. The oil obtained crystallized after 2 days' standing in a desiccator, and was recrystallized from benzene. Yield 77 % of the theoretical. M.P. 101°; a mixture with a sample prepared by the action of acetyl chloride showed no depression of M.P. (Found: C, 69·64; H, 5·35 %. $C_{11}H_{10}O_3$ requires C, 69·47: H, 5·30 %.)

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CXCI. STUDIES ON THE CHOLESTEROL CONTENT OF NORMAL HUMAN PLASMA

VIII. A NOTE ON THE EFFECT OF ANTICOAGULANTS

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SPERRY & SCHOENHEIMER [1935] concluded that for the determination of blood cholesterol, oxalated plasma was unsuitable. In one case they estimated the cholesterol contents of serum and oxalated plasma and found 409·5 and 300 mg. per 100 ml. respectively. They then instituted further comparisons and though they did not report finding any other differences of this degree, they stated that "the average difference between the amounts of total cholesterol in heparinized and oxalated plasmas was 15·3 % of the former ". They also quoted the work of Schmidt [1935] who found that the corresponding differences in the amount of phospholipid as estimated in heparinized and oxalated plasma averaged 10 % of the former.

There can be little doubt that the chief explanation of these differences is the effect of the oxalate addition in producing some shrinking of the red corpuscles; the fluid so lost diluted the plasma. This effect was noted by Gram & Norgaard [1923], and Eisenman *et al.* [1925] estimated this shrinkage by determining the serum protein after the defibrination of the blood with and without oxalate addition. They calculated the relative change in serum volume from the oxygen capacity and cell volume on the one hand, and from the protein content on the other. Both these methods demonstrated that the addition of oxalate to the blood caused a relative increase of plasma volume averaging 6 %. Eisenman [1927] showed that the addition of neutral potassium oxalate to the extent of 0·17–0·2 % of the whole blood caused a reduction of cell volume averaging 2 vol. % of the whole blood, which corresponds to a dilution of the plasma averaging 3·5 %. She also showed that with higher concentrations of oxalate, up to 0·45 %, there might be a shrinkage of the corpuscles up to 7·4 vol. % of the whole blood, equivalent to a plasma dilution of about 12 %.

Wintrobe [1931-2], in a series of haematological studies, stated that heparin is the ideal anticoagulant as it does not affect the size of the red corpuscles, but that it cannot always be depended upon unless the extremely expensive purified material is employed. He noted that there was a shrinkage of 8·2 % of the corpuscular volume in oxalated blood as compared with heparinized blood, and consequently he used the factor 1·09 to obtain the real corpuscular volume from the value obtained by using oxalated blood. Now the average corpuscular volume is 42 % of the whole blood, so that a shrinkage of 8·2 % of the volume would represent the addition of 3·44 vol. of fluid to 58 vol. of plasma, i.e. there would be a dilution of the plasma by approximately 6 %. It is interesting to note that this figure is the same as that determined by Eisenman *et al.* [1925].

Wintrobe's estimations of the change of corpuscular and plasma volumes by relatively simple physical methods are likely to be more accurate than the estimation of either cholesterol or phospholipid in the plasma by any method. As Sperry & Schoenheimer's results showed differences more than twice as large as the probable degree of plasma dilution, we thought that it would be of interest to make a further series of determinations.

Table I. *Comparison of the cholesterol content of heparinized and oxalated plasmas*

Series	Cholesterol, mg. per 100 g. plasma			% of total as ester	Mean values			
	Free	Ester	Total		Free	Ester	Total	
D 1:								
Heparin	47	135	182	74·2	—	—	—	
	45	134	179	74·9	46	134·5	180·5	
% difference	4·3	0·7	1·7	—	10·9	1·1	3·6	% diminution produced by oxalate
Oxalate	42	132	174	75·8	—	—	—	
	40	134	174	77·0	41	133	174	
% difference	4·9	1·5	0	—	—	—	—	
D 2:								
Heparin	34	118	152	77·6	—	—	—	
	34	118	152	77·6	34	118	152	
% difference	0	0	0	—	4·4	3·0	3·3	% diminution produced by oxalate
Oxalate	32	115	147	78·2	—	—	—	
	33	114	147	77·6	32·5	114·5	147	
% difference	3·1	0·9	0	—	—	—	—	
D 3:								
Heparin	32	120	152	78·8	—	—	—	
	33	116	149	78·1	32·5	118	151·5	
% difference	3·0	3·3	2·0	—	4·6	1·7	3·0	% diminution produced by oxalate
Oxalate	31	119	150	79·6	—	—	—	
	31	113	144	78·6	31	116	147	
% difference	0	5·0	4·0	—	—	—	—	
H 1:								
Heparin	71	183	254	72·1	—	—	—	
	72	180	252	71·3	71·5	181·5	253	
% difference	1·4	1·6	0·8	—	3·5	5·5	5·3	% diminution produced by oxalate
Oxalate	68	170	238	71·4	—	—	—	
	70	173	243	71·3	69	171·5	239·5	
% difference	2·9	1·8	2·0	—	—	—	—	
*H 2:								
Heparin	70	187	257	72·9	—	—	—	
	69	186	255	73·0	69·5	186·5	256	
% difference	1·4	0·5	0·8	—	2·9	5·4	4·7	% diminution produced by oxalate
Oxalate	68	176	244	72·6	—	—	—	
	67	177	244	72·2	67·5	176·5	244	
% difference	1·5	0·6	0	—	—	—	—	

* These results are expressed as mg. cholesterol per 100 ml. plasma.

Cholesterol was determined in heparinized and oxalated plasmas by the method described by us [Gardner *et al.* 1938]. Blood was taken from normal individuals or from patients who were unlikely to have any disturbance of their

cholesterol metabolism. Each specimen was divided into two portions of which one was treated with neutral potassium oxalate to the extent of 0·2 g. per 100 ml., and the other with heparin (Hyson, Westcott and Dunning's preparation) to the extent of 1 mg. per 5 ml. of blood. In all experiments, duplicate determinations were made. Cholesterol is expressed in mg. per 100 g. of plasma except in one series (H 2) where determinations were made on 10 ml. portions of plasma, and hence the results are expressed as mg. cholesterol per 100 ml. plasma.

The average diminution of total cholesterol content produced by oxalating plasma was 3·8 %, whereas the average difference between duplicate determinations of total cholesterol was only 1·1 %. As we do not yet understand the significance of much greater variations from the average normal, this reasonably constant error of 4 % is not a very grave matter.

SUMMARY

1. Addition of neutral potassium oxalate to blood leads to a small shrinkage of the erythrocytes, with a consequent slight dilution of the plasma.

2. In cholesterol estimations on heparinized and oxalated plasma there was found to be a reduction of concentration produced by neutral potassium oxalate of about 4 %. This figure is of the same order as the degree of shrinkage of the red cells.

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CXCII. CHANGES IN THE LIVERS OF MICE AFTER ADMINISTRATION OF 3:4:5:6-DIBENZCARBAZOLE

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THE known carcinogenic hydrocarbons produce primary tumours at the site of application [cf. Cook *et al.* 1937] and one of them at any rate (1:2:5:6-dibenzanthracene) increases the incidence of lung cancer in those strains of mice in which this condition occurs spontaneously [Lynch, 1935; Andervont, 1935]. The feeding [Sasaki & Yoshida, 1935] or injection [Shear, 1937] of 4'-amino-2:3'-azotoluene induces malignant changes in the livers of rats and mice. This compound, however, does not have any marked carcinogenic action at the site of administration. 3:4:5:6-Dibenzcarbazole produces sarcomata as a result of subcutaneous injection into rats and epitheliomata and bile duct hypertrophy resembling cholangiomata when it is painted twice weekly on the skin of mice [Boyland & Brues, 1937]. Strong *et al.* [1938] found that the injection of 3:4:5:6-dibenzcarbazole into mice of the C.B.A. strain, in which hepatoma occurs spontaneously, increased the incidence of these tumours.

As 3:4:5:6-dibenzcarbazole produces bile duct proliferation and neoplastic changes in the livers an attempt has been made to correlate the histological appearances with certain chemical changes. This compound is much more toxic to mice than carcinogenic hydrocarbons such as 1:2:5:6-dibenzanthracene or methylcholanthrene.

Methods. Mixed stock mice previously fed on Purina Fox Chow for a week received a single intraperitoneal injection of 3:4:5:6-dibenzcarbazole dissolved in olive oil. Mice weighing less than 16 g. were not used as it was found that the compound was more toxic to young mice. During the experiment the diet consisted entirely of Fox Chow. At varying intervals after the injection mice were weighed, killed and their livers removed. A small portion of the liver was taken for histological section, and the remainder was investigated chemically. In some cases a small piece was also taken for estimation of the glycolysis and respiration in the Warburg apparatus.

The liver tissue, after weighing, was ground in a centrifuge tube with 3 vol. of 5 % trichloroacetic acid, the suspension centrifuged and an aliquot portion of the supernatant fluid removed for determination of ascorbic acid and glutathione as described by Mawson [1935]; the ascorbic acid was first titrated with phenol-indo-2:6:dichlorophenol and then the glutathione with iodine. The trichloroacetic acid precipitate was saponified with KOH on a boiling water bath and the "fat" estimated by the Liebermann method as described by Leathes & Raper [1925]. According to Dulière and Minne [1937] cholesterol is completely removed from blood serum by precipitation with trichloroacetic acid and we have found that estimations of the fat content on the trichloroacetic acid precipitate of liver give the same values as estimations on fresh tissue. The cholesterol in the

"fat" was determined by a modification of the method of Schoenheimer & Sperry [1934] for blood; the material was dissolved in 5 ml. acetone-alcohol (1:1) and 2 ml. 0·5% digitonin in 50% alcohol were added. The solution was allowed to stand overnight and next day the precipitate was centrifuged and washed once with acetone-ether (1 : 2) and twice with ether. The last traces of ether were removed in an oven at 40' and the precipitate dissolved in 4 ml. glacial acetic acid. 8 ml. acetic anhydride and 0·4 ml. conc. H_2SO_4 were added and the colour which developed was compared in a colorimeter with that of a cholesterol standard, using a red screen.

Results

Series 1. In the first experiment 0·5 mg. 3:4:5:6-dibenzcarbazole was given to each mouse, and there was a considerable mortality during the week following the injection. Of a series of 38 mice, 10 were alive on the 3rd day after injection: 4 of these were killed and only 3 of the remaining 6 were alive on the 5th day. Many of the livers were very pale and appeared fatty. The most striking chemical change was in the glutathione (Fig. 1): the amount in many cases was definitely
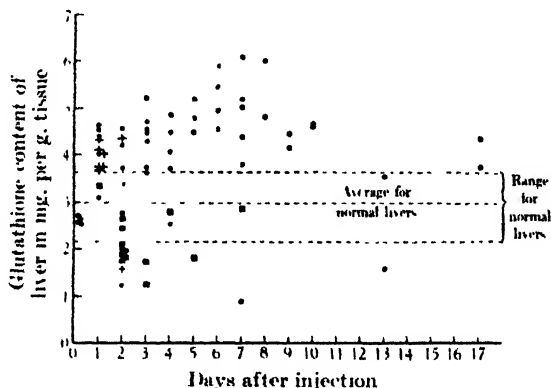
Fig. 1. The glutathione content of livers of mice injected with 0·5 mg. 3:4:5:6-dibenzcarbazole. • Represents liver of normal histological appearance. ■ Represents liver with advanced necrosis. + Represents liver with early necrosis.

increased and in 3 mice was twice the normal average. This average (2·91 mg. per g. with a standard deviation ±0·37) was obtained from 30 untreated animals. Eight control mice injected with 0·5 ml. olive oil and killed 3–10 days afterwards had an average liver glutathione of 2·68 with a standard deviation ±0·23. In all the 4 mice killed 3–4 hr. after injection with 3:4:5:6-dibenzcarbazole the amount was within the normal range. At the end of 24 hr. the rise was quite definite and the highest values occurred between the 3rd and 8th days. This rise in glutathione, however, did not occur invariably. Among 16 mice killed 48 hr. after injection 4 were within the normal range and 8 were below it. But 31 out of 40 mice killed after 48 hr. showed a glutathione content above the highest limit of the normal range.

The ascorbic acid content of these livers showed no significant change. There was great variation among apparently normal mice (0·152–0·465 mg. per g.) and 59 out of 67 treated animals (86%) were within this range. In all cases where the glutathione was very low the ascorbic acid was below the normal average. Fat and cholesterol estimations were not carried out in this experiment.

Histological examination of paraffin sections (special methods for staining fats were not used) suggested that many of the livers were either very fatty or contained glycogen, in agreement with the pale colour seen with the naked eye. However, fat or glycogen appeared to have been present in sections of the livers of untreated mice which had been fed on Fox Chow and, as it is impossible to estimate the amount of fat histologically, no conclusion could be drawn. No proliferation of the bile ducts was observed in these mice of which none lived more than 17 days. The most striking feature observed histologically was the differentiation of the liver lobule into an inner zone of well stained cells surrounding the central vein and an outer zone of large degenerate cells which appeared to have contained fat or glycogen. This zoning was observed in less than one-third of the mice and in some of these was very slight. The glutathione content of these has been indicated in Fig. 1, which shows that most of the slightly necrotic livers occurred 24 hr. after the injections and the glutathione in these was above the normal limit. Seven out of 13 livers showing toxic necrosis and degeneration were in mice which had been injected 48 hr. previously, and all of these (except one at 24 hr.) contained less than the normal average of glutathione, and the majority (8 out of 13) less than the lowest normal limit. The most advanced necrosis was seen in two livers at the 4th and 7th days.

None of the livers of mice killed 8 days or more after injection showed necrosis or hepatitis indicating that recovery and regeneration occurred. Many of these livers showed signs of regeneration and growth. With the completion of recovery the glutathione content returned almost to the normal value.

Determinations of the respiration and glycolysis (Table 1) show that the four livers with necrotic areas on which these determinations were made had low values for respiration, with Q_{O_2} ranging from $-2\cdot3$ to $-7\cdot6$ instead of the

Table I. *Livers of mice injected with 0·5 mg. 3:4:5:6-dibenzcarbazole*

Time after injection days	Glutathione mg. per g.	Ascorbic acid mg. per g.	Metabolism		Histological appearance
			Respiration Q_{O_2}	Glycolysis $Q_L^{N_2}$	
2	1·22	0·135	− 3·4	+1·4	—
2	3·73	0·310	−14·3	+4·1	—
2	2·44	0·152	− 7·6	+2·0	Intense hepatitis
2	2·02	0·191	− 3·6	+1·6	„
3	1·23	0·200	− 2·3	+1·3	„
3	4·47	0·428	−10·8	—	—
3	4·52	0·333	− 9·1	+3·4	—
4	3·70	0·258	− 9·7	—	—
6	4·97	0·285	− 7·0	+3·2	—
6	5·98	0·250	−10·8	+2·3	—
7	0·86	0·106	− 9·2	+6·1	—
7	6·08	0·330	− 5·0	+3·1	—
7	2·82	0·250	− 3·7	+1·3	Intense hepatitis
8	4·82	0·293	− 6·9	+2·5	—
9	4·17	0·292	− 8·6	+1·4	—
10	4·66	0·099	− 7·0	—	—
10	4·62	0·149	− 3·3	+3·9	—
13	1·59	0·038	− 3·2	—	—
13	3·52	0·460	−11·7	—	—
17	4·37	0·234	− 4·8	+3·8	—
17	3·72	0·148	− 2·3	—	—

normal values of -10 to -12. The metabolism of the other livers is on the whole low but irregular.

Series 2. The mortality in the first experiment (Fig. 1) was high; no mice were examined after 17 days, and therefore the chance of any development of

neoplastic changes in the liver was small; hence the dose was reduced to 0·25 mg. 3:4:5:6-dibenzcarbazole per 20 g. mouse (Fig. 2). Ascorbic acid, glutathione, fat and cholesterol were estimated on all livers in this experiment. Many died (of a series of 65 mice 28 (43 %) were alive 39 days after injection) but many lived for long periods and those which survived for 200 days appeared healthy. One mouse killed on the 176th day after injection had a spindle-celled sarcoma at the site of injection. The livers of many killed after the 40th day showed a nodular appearance and in others the lobes seemed to be swollen and often two or more lobes appeared fused.
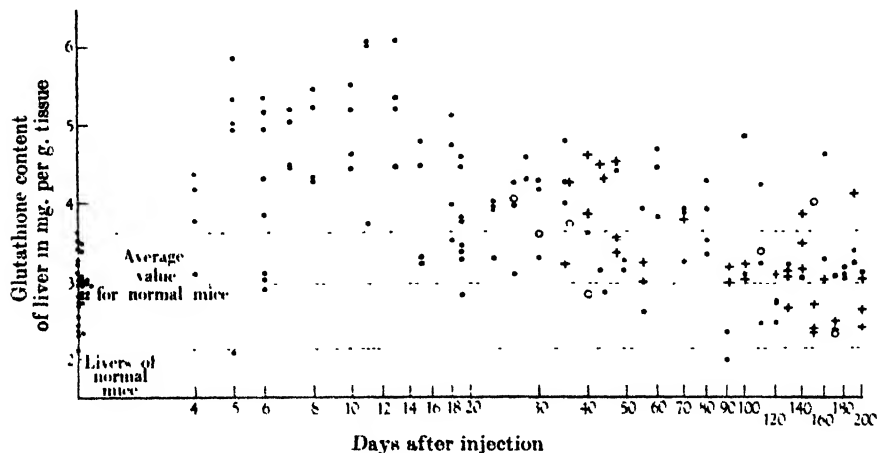
Fig. 2. The glutathione content of livers of mice injected with 3:4:5:6-dibenzcarbazole (12·5 mg. per kg. body wt.). ● Represents liver with normal appearance of bile ducts. o Represents liver with early bile duct proliferation. + Represents liver with advanced bile duct proliferation.

The glutathione contents of the livers are shown in Fig. 2. Again a very distinct increase was observed which was much more consistent than in the earlier experiment. Only three values below the normal average occurred in the 40 days following injection and two of these were close to the normal average. The glutathione content was greatest during the first 20 days and then fell off gradually. Among 34 livers examined between the 120th and 200th days there were only four values above the normal range. The ascorbic acid content lay almost wholly within the wide normal range. The cholesterol and fat contents also remained approximately normal except for a few isolated cases. The cholesterol content varied from 0·216 to 0·645 % except for two livers with 0·169 and 1·075 % cholesterol. The liver fat content ranged from 2·1 to 8·6 % with two exceptions of 1·6 and 11·2 %. The variations in fat and cholesterol could not be correlated with histological appearance. The hepatitis and necrosis which were so striking in the first series were seen in only 7 mice. The instances of early bile duct proliferation and advanced bile duct hypertrophy (the distinction between these two conditions is quite arbitrary) are indicated in Fig. 2. The advanced biliary hypertrophy resembles cholangioma but as transplantation experiments were not performed it is not possible to decide whether this type of growth is malignant or not. No cases of advanced hypertrophy occurred before the 35th day and 33 of 83 mice (40 %) killed after this time showed definite bile duct proliferation. One mouse on the 26th day and another on the

30th day showed indications of the development of this condition. Cholangiomatous growth was observed in livers both with normal and with increased glutathione.

Table II. *Livers of mice injected with 3:4:5:6-dibenzcarbazole* *(12·5 mg. per kg. body weight)*

Time after injection days	Glutathione content mg. per g.	Ascorbic acid content mg. per g.	Metabolism		Histological appearance
			Q_{O_2}	Q_L^N	
10	5·18	0·37	− 8·3	4·9	—
90	1·98	0·51	− 13·5	1·2	--
90	2·33	0·29	− 13·8	3·5	---
163	2·90	0·56	− 8·9	1·5	--
163	3·40	0·42	− 10·4	4·6	—
164	4·05	0·41	− 5·6	2·6	
164	3·54	0·41	− 8·1	2·1	Advanced bile duct hypertrophy
164	3·54	0·37	− 7·7	4·4	--
172	3·35	0·25	− 14·7	2·0	Advanced bile duct hypertrophy
172	3·55	0·31	− 9·0	2·1	,,
172	3·23	0·36	− 9·6	2·4	,,
176	2·72	0·30	- 7·8	1·1	,,
176	2·15	0·33	− 6·2	4·3	,,
176	4·50	0·17	-- 6·8	2·7	,,
176	3·65	0·27	− 7·7	1·3	,,

Table III

Time after injection days	Wt. before injection g.	Wt. after injection g.	Wt. of liver g.	Glutathione mg. per g.	Ascorbic acid mg. per g.	Fat %	Cholesterol %

Livers of mice injected with 1 mg. methylcholanthrene

4	23	17·0	0·89	2·16	0·190	3·4	0·37
4	27	19·5	1·12	2·60	0·331	3·8	0·31
4	27	19·5	1·28	2·27	0·336	4·1	0·30
4	23	20·0	1·32	2·60	0·315	4·4	0·27
8	27	21·0	1·18	2·23	0·304	3·6	0·38
8	25	20·5	1·00	2·79	0·325	4·8	0·35
8	18	17·5	1·02	3·29	0·375	3·6	0·28
8	22	17·5	1·10	3·30	0·358	3·5	0·34
12	24	17·5	0·90	2·77	0·272	3·1	0·32
12	21	16·5	0·78	2·40	0·281	3·1	0·40
12	26	22·0	1·05	2·88	0·380	4·1	0·33
12	18	16·5	0·98	3·77	0·370	3·8	0·31
16	20	28·0	2·30	3·37	0·422	2·9	0·31
16	21	27·5	2·16	2·72	0·411	3·0	0·28
16	19	22·0	1·73	3·00	0·400	3·2	0·38
16	16	21·5	1·85	3·80	0·450	4·0	0·32
20	—	26·0	1·66	3·12	0·406	4·0	0·27
20	18	20·0	1·34	2·81	0·433	3·9	0·26
20	—	16·0	0·84	2·50	0·282	3·6	0·32
20	—	20·0	1·46	2·82	0·502	3·8	0·28

Livers of mice injected with 2 mg. 1:2:5:6-dibenzanthracene

3	—	—	—	3·00	0·217	2·9	—
4	—	—	—	2·78	0·324	3·4	—
4	—	—	—	3·47	0·291	3·5	—
6	—	—	—	2·50	0·272	3·6	—
6	—	—	—	2·58	0·299	3·0	—
7	—	—	—	1·76	0·328	3·5	—
7	—	—	—	3·63	0·230	5·0	—
10	—	—	—	3·37	0·239	3·5	—

The metabolisms of the livers of some of the mice were determined and the results obtained are given in Table II. The metabolism of livers with advanced biliary hypertrophy is of the same order as that of normal livers.

Series 3. Eight mice which were injected intraperitoneally with 2 mg. 1:2:5:6-dibenzanthracene in olive oil and killed 3–10 days afterwards showed no significant changes in the glutathione, ascorbic acid or fat content of the liver (cf. Table III). No histological examination was carried out.

Twenty mice were injected intraperitoneally with 1 mg. methylcholanthrene dissolved in 0·25 ml. lard per 20 g. mouse and killed at intervals up to 20 days after the injection. The livers of these animals appeared normal except for numerous adhesions. The analytical results obtained on these livers are shown in Table III: no significant changes were observed and histological examination of all the livers revealed no abnormalities.

<h2 style="text-align:center">DISCUSSION</h2>

Considerable changes in the livers of mice may occur as the result of a single intraperitoneal injection of 3:4:5:6-dibenzcarbazole (0·25 or 0·5 mg.). Boyland & Brues [1937] found that rats receiving twice weekly subcutaneous injections of 1 mg. of this compound lived for a long time and showed no liver lesions although spindle-celled sarcomata developed at the site of injection, while mice receiving this treatment died after one or two injections. In our own experiments the mortality was also high in mice which were given a single dose of 0·5 mg. 3:4:5:6-dibenzcarbazole and striking histological changes (hepatitis and necrosis) were observed in the livers of many of those killed during 7 days following the injection. It would appear that 3:4:5:6-dibenzcarbazole is more toxic to mice than to rats and this may possibly be associated with its effect on the liver in the mouse With 0·25 mg. of the compound the mortality was much less and few cases of hepatitis were observed.

The first definite proliferation of bile ducts was observed 35 days after injection of 0·25 mg. although the first indication of such development was seen at the 26th day. These times agree very well with those at which Boyland & Brues observed similar biliary proliferation in mice painted twice weekly with the same compound.

The glutathione content of the liver had increased 24 hr. after injection and was at its maximum during the first 20 days, after which it decreased. Since the ascorbic acid, fat and cholesterol contents remained approximately normal we consider that this is a true increase in glutathione and is not due to a decrease in the water content of the liver. The glutathione content was at its maximum before any neoplastic change was observed (Fig. 2). Some of the "cholangiomata" were seen in livers with an increased glutathione content but most of them occurred when the glutathione had returned to normal.

In the first experiment the livers with highest glutathione content were found after the period when the histological changes were most marked. High glutathione content was often present in those livers in which regeneration was taking place. In about 10 days when the regeneration was complete the glutathione content fell again. In the second experiment, in which the dose of 3:4:5:6-dibenzcarbazole was less, very high values for glutathione content were found during the first 20 days and during this time recovery from the injection with regeneration of cells was probably taking place. Although in many cases the livers were greatly enlarged it was not possible to correlate liver weight with glutathione content. The results indicate that the 3:4:5:6-dibenzcarbazole has

a toxic action which is followed by regeneration accompanied by an increase in glutathione, and at a later stage bile duct hypertrophy takes place.

The decrease in glutathione content which was observed (Fig. 1) in certain mice receiving 0·5 mg. 3:4:5:6-dibenzcarbazole was accompanied by a decrease in ascorbic acid and both the macroscopic appearance of the livers and the study of histological sections suggest that this may have been due in part at least to an accumulation of fat.

1:2:5:6-Dibenzanthracene, which produces hepatoma in mice of the C_3H strain [Andervont, 1937] but has not been shown to produce proliferation of bile ducts, and methylcholanthrene, which is not known to produce either of these changes, did not affect the glutathione, ascorbic acid or fat content of the livers of mice.

SUMMARY

A single intraperitoneal injection of 0·25 mg. 3:4:5:6-dibenzcarbazole per 20 g. mouse caused an increase in the glutathione content of the liver which was maximal during the first 20 days. This preceded a considerable proliferation of bile ducts which occurred in 40 % of the mice examined between the 35th and 200th days. When 0·5 mg. was given there was a similar increase in glutathione content in the majority of the mice but in a few there was a decrease which in most cases was accompanied by a partial necrosis of the liver lobules. This increase in glutathione was not observed in mice injected with 1:2:5:6-dibenzanthracene or with methylcholanthrene. No change in the ascorbic acid or cholesterol of the liver occurred with any of these carcinogenic compounds.

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CXCIII. THE EFFECT OF VITAMIN E DEFICIENCY ON THE RAT

I. DURATION OF GESTATION

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It has been known for some time [Kirkham, 1916; Brambell, 1937] that if small rodents or shrews are mated during the *post partum* oestrus so that lactation and gestation are simultaneous, then provided that the number of young suckled exceeds a minimum, which is characteristic for each species and is 5 for the rat, gestation is prolonged. There is no direct correlation between the duration of gestation and the number of young suckled, but a rat suckling 9 young may have a gestation period lasting 10 days beyond the normal period.

It has been noticed, in the course of a number of vitamin E assays, that among the proved sterile animals which had been given a small dose of vitamin E, gestation was often prolonged beyond the normal 22 days characteristic of our stock. This prolongation was so consistent among the rats suffering from severe vitamin E deficiency that a further investigation of the problem was undertaken, which has already been the subject of a preliminary communication [Barrie, 1937, 3].

Two diets were used throughout the experiment:

Diet E. 5.	Fat-free casein	2000 g.
	Rice starch	6000 ,,
	Lard	800 ,,
	Cod liver oil	200 ,
	Salt mixture	500 ,,
	Yeast extract	180 I.U. vitamin B_1 per 100 g. diet

This diet contains no detectable amount of vitamin E and 635 animals fed on it from weaning all resorbed in their first gestation.

The second diet, E. 4, differed from E. 5 in that light white casein was used instead of fat-free casein. However, animals fed on it were usually capable of producing two litters before the resorption typical of vitamin E deficiency occurred. That the E. 4 diet contained inadequate vitamin E, even for the first of these litters, was shown by the fact that the young produced became abnormal and usually died within 23 days after birth [Barrie, 1937, 1, 2].

In the experiments which are reported here, all the test animals were mated with males which had been fed on a diet rich in vitamin E and been proved fertile. Successful implantation of the ova was verified by the finding of the placental sign (detection of blood in the vagina) between the 9th and 13th days after mating. A daily record of the weight and condition of the animals was kept from the occurrence of the placental sign and was continued either until the birth of the young, or until resorption was so advanced that the weight had fallen considerably. In those cases in which young were born, a daily record was kept of the weight and condition of the litter.

The test animals were divided into 5 groups:

1. *Negative controls.* These were not specially selected and were the sterile and untreated representatives of the colony of vitamin E-free rats. These rats were fed on the vitamin E-free diet E. 5 from weaning, and in spite of positive mating followed by successful implantation, the foetuses were resorbed *in utero* in the first gestation.

2. *Animals severely deficient in vitamin E.* This was a group of 22 rats fed on the E. 4 diet until approximately 1 month before mating when they were transferred to diet E. 5. These animals usually produced a living litter but were never able to rear it.

3. *Animals slightly deficient in vitamin E.* This was a group of 40 fed on the diet containing a small but still inadequate amount of vitamin E (diet E. 4). Although able to produce living litters, they were seldom able to rear them.

4. *Positive controls.* This was a group of 27 fed on the vitamin E-free diet (E. 5), but on mating they were given a large dose of a vitamin E concentrate. The adequacy of the dose was shown by the ability of the doe to rear her litter.

5. *Stock rats.* This was a group of 50 rats selected at random from our normal breeding stock. They were fed on a diet containing an adequate amount of vitamin E. These animals all reared their litters and the few deaths which occurred among the young were usually due to the mother becoming fierce and killing them.

Table I

Group	No. rats in group	Length of gestation (days)			No. young born	No. born dead	No. young reared
		Average	Longest	Shortest			
2	22	25·3 (corrected 24·8)	32	23	71	43	1
3	40	23·0 (corrected 22·5)	25	19	242	24	62
4	27	23·04 (corrected 22·54)	25	22	175	17	119
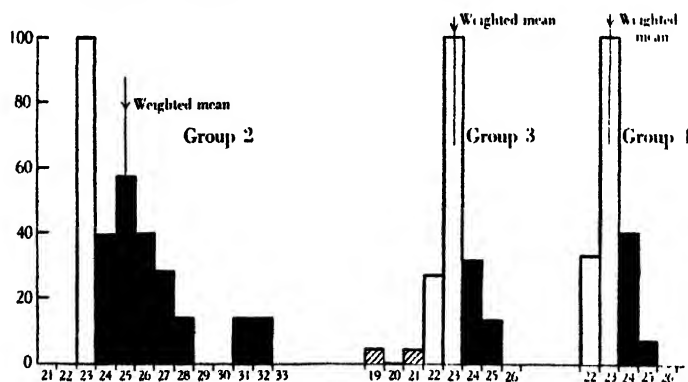
Fig. 1. Plotted to show the relative distribution of the gestation period. The largest group was arbitrarily selected as 100 in each case. The hatched columns represent gestations which were shorter than normal, the hollow columns those of normal duration, and the black columns those in which the duration was beyond the normal range.

Table I gives the length of gestation and the live and dead litter rates in animals of groups 2, 3 and 4. Since the animals were weighed and examined only once daily, at approximately 11 a.m. on each day, the litters born were anything up to 24 hr. old when first observed. In order to correct for this, one half day has been deducted from the average gestation period for each group. It will be seen that the positive controls had a gestation period varying between 22 and 25 days with an average of 23 days, or 22·5 after making the correction. This

period is the same as that of our normal breeding stock (see also Hain [1934]). The normal range must, however, be taken to lie between 21 and 23 days, as a variation of one day could not be considered abnormal.

The animals which were suffering from a slight deficiency of the vitamin (group 3) had a gestation period of 19–25 days, 67·5 % of them falling within the normal range of 22–23 days; those of group 2, which were suffering from a much more severe deficiency of vitamin E, had a gestation period which lasted as long as 32 days in one case, and the duration was normal in only 31·82 % of the animals. Fig. 1 shows these results scaled down so that the relative spreads in each group can be compared. The weighted mean of groups 3 and 4 is 23 days, while that of group 2 is 25·3 days (both uncorrected).

These results give a definite indication that the length of gestation bears an inverse relation to the amount of vitamin E in the animal's diet.

Although the majority of the animals of group 3 which were suffering from a slight deficiency of vitamin E had a gestation period of normal duration, their deficiency of vitamin E was shown by the low survival rates of the litters.

Table II

Mother	No. of litters averaged	Av. no. born per litter			Av. no. reared
		Total	Born alive	Born dead	
Group 5	50 First litter in each case	7·84	7·84	0·00	7·24
Group 4	27	6·48	5·87	0·63	4·40
Group 3	40	6·05	5·99	0·06	1·55
Group 2	22	3·23	1·28	1·95	0·04
Group 1	635 pregnancies	0·00	0·00	0·00	0·00

Table II gives the average number of young born to animals in each of these groups, and again a difference is seen: the animals receiving adequate vitamin E (group 4) had litters averaging 6·5 young with an average of 0·6 still-births per litter. Those receiving less vitamin E (group 3) had litters of approximately the same number, but the still-birth rate was slightly higher and the litters were not reared, while the animals of the second group which had not received any vitamin E for a month before mating had very small litters averaging 2·14 live- and 1·27 still-births per litter. In spite of the smaller number born, the mothers were unable to rear them. Among our normal breeding stock, which is fed on a diet rich in vitamin E, we find that the average number of young born is 7·84 and in the 50 selected at random there were no still-births. Among these normal breeding stock rats there are a few which never give birth to more than 5, even in the first gestation, while others have as many as 14. Old breeding rats frequently have small litters, but if the number born is fewer than 5, we consider the animal unsuitable for breeding. Hence it may be concluded that if a vitamin E-deficient rat is dosed with vitamin E and has a litter of 5, the dose given is not necessarily smaller than that which produced 7 in another rat, but if the number in the litter is less than 5 and the animal is young and in good condition, there is an indication that the dose given was inadequate; this supposition is borne out by the fact that such small litters are seldom reared by vitamin E-deficient rats.

Fig. 2 shows typical weight curves (*A*) of a resorbing rat, (*B*) of a rat given a large dose of vitamin E and (*C*) of a rat suffering from severe vitamin E deficiency. These three rats were chosen because they were all at approximately the same weight on the 13th day of pregnancy. The three curves are very similar

until the 16th day, from which date the dosed rat (136) gained weight until full term. This weight curve is typical of those given by the 27 positive controls. The resorbing rat and the rat partially deficient in vitamin E ran together until the 17th day; the latter rat then started to gain weight more rapidly until the birth of the litter on the 32nd day of pregnancy.
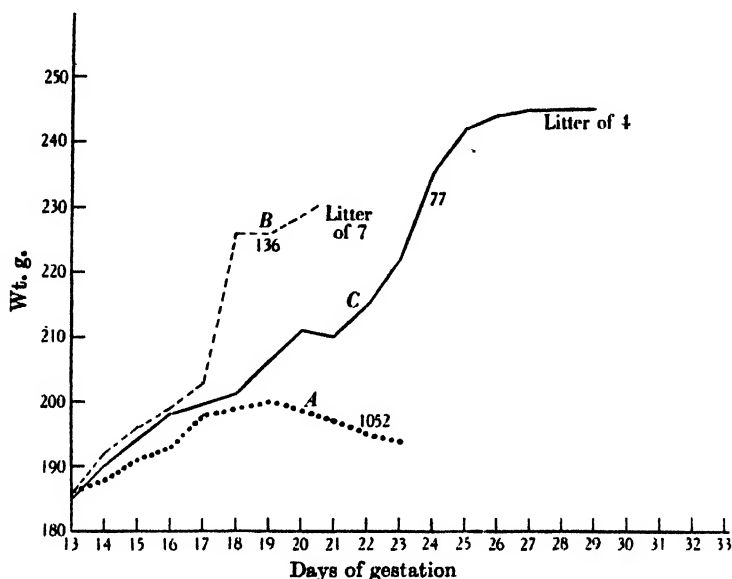
Fig. 2. Rat 136 dosed with vitamin E; rat 77 given inadequate vitamin E; rat 1052 given no vitamin E.

A pregnant rat deficient in vitamin E, which is also receiving a diet containing no vitamin E, gains weight slowly from the time of implantation until about the 15th day; after this stage the weight gradually decreases, and there may be a haemorrhage, possibly comparable with abortion, at any time from the 16th day onwards (Fig. 2, rat 1052). Only one rat has been taken as an example, as the rats become pregnant at different weights and it would not be desirable to make a composite curve. The curve for this single rat agrees with that of Evans [1927] and is typical of over 800 obtained during this and other experiments. Placentation is often early in these rats and if it is detected on the 9th day of gestation and is followed by continuous bleeding it is fairly certain that resorption will follow; in fact, the time and type of placental sign found give a fairly accurate forecast of the course of pregnancy. Tables III, IV and V illustrate this point.

Rat 697 is representative of group 4, rat 658 had very little reserve of vitamin E and is therefore comparable with rats of group 2, while rat 115 had been fed on the vitamin E-free diet from weaning and belonged to the negative control group 1. Comparison of these three records shows that when pregnancy takes a normal course there is very little bleeding.

To the rat whose gestation period lasted 32 days, only 4 young were born and these were well above the average birth weight, weighing 8 g. each instead of the normal 4·5 g. In a few cases, animals have died or have been killed at some stage during the pregnancy and the *post mortem* findings have given the explanation of this prolonged gestation. One of these animals was 19 days

Table III. *Rat 697 fed on vitamin E-free diet, but given a dose of 2·5 ml. vitamin E concentrate on mating. This concentrate was known to be active in a dose of 1·5 ml.*

Stage of pregnancy days	Wt. g.	Vaginal smear
13	184	Faint trace of blood
14	184	Mucus and blood
15	186	Mucus and blood
16	189	No blood
17	191	No blood
18	196	No blood
19	202	No blood
20	211	Trace of blood. Foetuses could be felt
21	220	More blood than preceding day
22	222	Unchanged
23	229	Occult blood no fresh blood
24	Litter of 6	—

Table IV. *Rat 658 fed on vitamin E-rich diet for 4 months then on vitamin E-free diet for 3 months before mating. The animal had practically run out of vitamin E*

Stage of pregnancy days	Wt. g.	Vaginal smear
10	188	Trace of blood
11	189	Trace of blood
12	Not examined	—
13	187	Trace of blood
14	195	Mucus and blood
15	196	Very faint trace of blood
16	200	Fresh blood
17	202	Very faint trace of blood
18	206	Quite a lot of blood
19	Not examined	—
20	203	Mucus
21	212	Mucus
22	216	Trace of blood. Foetuses felt
23	221	Faint trace of blood
24	224	Faint trace of blood
25	Litter of 3 (2 dead)	—

Table V. *Rat 115 fed on vitamin E-free diet from weaning. First pregnancy*

Stage of pregnancy days	Wt. g.	Vaginal smear
10	180	Trace of blood
11	180	Trace of blood
12	Not examined	—
13	183	Trace of blood
14	180	Large amount of blood
15	186	Large amount of blood
16	181	Fresh blood
17	178	Bleeding profusely
18	176	Blood and mucus
19	Not examined	—
20	175	Bleeding profusely
21	175	No blood
22	176	Fresh blood
	Litter resorbed	

pregnant when it died and the remains of 7 resorbing foetuses could be seen together with two apparently normal but very undersized ones which would probably have been born at some time after 21 days' gestation. Similar conditions have been seen in animals which have reached the 24th day of gestation. In a preparation made from a 23 days' pregnant vitamin E-deficient rat which had been given a small dose of vitamin E, resorption of three foetuses was well advanced in the right horn of the uterus, while in the left horn two others were developing, and one was probably being resorbed. This evidence, together with that of the weight curves (Fig. 2), shows that resorption of the litter is not an "all or none" process and that there are varying degrees of vitamin E deficiency. Partially deficient animals resorb some of their foetuses and develop the others so that the number carried to term is smaller than would normally be the case.

It seems probable that owing to the process of resorption, the development of the remaining foetuses is retarded and in consequence pregnancy is prolonged. This is shown very clearly in Fig. 2 where the weight curve of the rat which had a 32 days' gestation first follows the line of the resorbing rat and then leaves it to make normal daily increases in weight until the birth of the litter.

Similar prolongations of gestation have been observed before in the rat, the mouse and the shrew, but under very different circumstances. Kirkham [1916; 1918] found that this prolonged gestation in the mouse was associated with delayed implantation and that the retardation occurred in the blastula stage of the embryo. There was no evidence of resorption of any of the foetuses. The phenomenon is therefore hardly comparable with that observed in the vitamin E-deficient rat, in which implantation is often earlier than normal and is followed by resorption of some of the foetuses. The prolonged gestation in both these and the lactating animals is very probably due to some impairment of the normal function of the corpus luteum. It is here that a connexion between the two sets of results seems most probable, if the function of the anterior pituitary gland is controlled by vitamin E. This theory is strongly supported by the work of Rowlands & Singer [1936], who showed that the luteinizing activity of the pituitary of vitamin E-deficient rats is about half that of the normal rat. Verzár & Kokas [1931] showed that the coat of the vitamin E-deficient male rat changed to the type characteristic of hypophysectomized animals and later Verzár [1937] summarized the published work on vitamin E which gives support to this theory. Nelson [1933] found castration changes in the anterior pituitary of vitamin E-deficient male rats and Barrie [1937, 2] described degranulation of the acidophils and basophils of the anterior pituitary of the vitamin E-deficient female rat and ascribed the abnormal condition of the young born to partially vitamin E-deficient rats to anterior pituitary involvement. It can therefore be taken that the vitamin E-deficient animals used in these experiments, in which gestation was prolonged, were suffering from anterior pituitary deficiency. It seems probable that this deficiency was sufficiently severe to impair the function of the corpus luteum, although so far no morphological evidence of such impairment in vitamin E deficiency has been found.

SUMMARY

In the rat partially deficient in vitamin E the length of gestation bears an inverse relation to the amount of vitamin E in the diet. Such partially deficient animals are unable to develop all the ova implanted, with the result that some of the foetuses are resorbed *in utero*.

It is suggested that the prolongation of gestation caused by vitamin E deficiency is due to the resorption of some of the foetuses delaying the development of the others and that this condition is an indirect effect, through the ovary, of anterior pituitary deficiency.

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CXCIV. THE EFFECT OF VITAMIN E DEFICIENCY ON THE RAT

II. LACTATION

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THE accepted characteristic of vitamin E deficiency is resorption of the foetuses *in utero* before the completion of gestation. In ordinary circumstances, absolute deficiency of a vitamin must be a very rare occurrence and for this reason the characteristics of partial deficiency are probably more readily observed.

Vitamin E is in some way connected with the normal function of the anterior pituitary gland [Verzár, 1937: Barrie 1937, 1] and when one considers the diversity of function of this gland it is not surprising that the effects of even a partial deficiency of the vitamin are widespread.

If a female rat is fed on a diet which contains inadequate vitamin E, she may produce a living litter after mating with a normal male but is unable to rear it because the young become abnormal and die within 21 days of birth. This abnormality was described in an earlier paper [Barrie, 1937, 1] and an apparently similar condition was observed by Evans [1928] and found by him to be preventable if the young were dosed with wheat germ oil within 15 days of birth. In a preliminary communication [Barrie, 1937, 2] the result of an experiment was given in which it was found that this abnormality of the young, the onset of which occurred about 18 days after birth, was due to some deficiency in the mother's milk. The experiment has been repeated and confirmed.

Rats which had been proved to be sterile were mated and given a small dose of a vitamin E concentrate on the day of mating. The activity of the concentrate used had been previously determined and the dose given was known to be adequate for the production of a litter, but inadequate for the rearing of it. In one experiment the litter was exchanged for a normal stock litter born on the same day, in another experiment the exchange was made at 3 days, and in further experiments at 5 and 6 days after birth. In every case it was found that the animals fed by the vitamin E-deficient mother became abnormal, while those fed by the stock mother grew normally.

Full details of the first of these interchange experiments are given in Table I.

On the 16th day the young fed by the vitamin E-deficient rat were all definitely affected, although her own males were weaker than the females she was fostering. At this stage of the experiment the stock mother's own males were killed as she was a very small rat and a litter of 10 seemed too much for her; she was therefore left with only the fostered females from the vitamin E-deficient rat. At 17 days the young fed by the vitamin E-deficient rat were definitely abnormal; they were dragging their hind legs and their forepaws were contracted and on the following day the condition of the males was so bad that they were killed. The fostered females reached the same condition on the 21st day but the females born to the vitamin E-deficient mother and fed by the stock mother grew normally and were weaned when 21 days old.

Table I. *Rat 214, partially deficient in vitamin E; litter of 8, 3 ♂ 5 ♀; total wt. 43 g. 6 days after birth total wt. 77 g., all apparently normal. The 5 ♀ exchanged for 4 stock ♀ born on the same day; the vitamin E-deficient mother then had 4 stock ♀ and her own 3 ♂, the stock mother had 5 vitamin E-deficient ♀ and her own 5 ♂*

Days after birth	Fed by vitamin E-deficient mother								Fed by stock mother					
	Wt. ♂ g.			(Fostered) Wt. ♀ g.				♂	(Fostered) Wt. ♀ g.				
6	10	10	10	11	11	11	11	Not weighed	10	9	9	10	9
9	14	13	14	12	12	12	13		12	12	12	12	12
14	19	19	19	18	18	18	18	Smaller but more lively	17	18	18	17	18
16	20 20 20 One has weak hind legs Another has front paws contracted			20 19 20 20 Two have weak hind legs				♂'s killed because the litter was too large	17 18 19 16 18 Normal Normal				
17	22 22 20 Two very abnormal*			22 22 22 22 No change									
18	19 22 18 Condition much worse, killed			23 23 23 23 All have weak hind legs					18 20 20 18 18 20 All active and normal				
21				32 27 25 1 dead others very weak and dragging hind legs					25 26 27 24 27				
22				All dying, killed					28 28 31 26 29 Normal, weaned				

* Fur soft and cottony, hind legs dragging, fore feet contracted; animals very weak

A very similar result was obtained when the exchange was made at 5 days, but in this case the males and not the females were exchanged. The result was further confirmed by giving to another vitamin E-deficient mother 4 stock young in addition to her own 2. The young were 3 days old when given to the vitamin E-deficient mother and they and her own litter became abnormal and died when between 20 and 22 days old.

In a further case a vitamin E-deficient rat had a litter of 6 which died when 12 days old and the mother was given 2 stock rats, aged 8 days, to foster. Both of these were reared.

In a somewhat similar experiment a sterile vitamin E-free rat was dosed with vitamin E concentrate though it was still fed on the vitamin E-free diet. This rat had a litter of 8, 5 of which were born dead, and one of which died later, leaving 2 which grew very slowly and died when 16 days old. The mother was given 4 stock young aged 3 days which she fed for 8 days, then 2 of them were returned to their own stock mother and 3 other stock young, aged 4 days, were given to the vitamin E-deficient mother, who was thus fostering 2 stock rats aged 11 days and 3 aged 4 days. The older two developed normally, as did the two which had been returned to their stock mother after being fostered by the vitamin E-deficient mother for 8 days. The 3 younger animals fostered by the vitamin E-deficient rat developed the characteristic leg weakness when 18 days old.

The exchange experiments show that the milk of the vitamin E-deficient rat is lacking in some substance essential for the normal growth and development of the suckling young, but that if the young are fed on normal rat milk until they

are 8 days old they obtain sufficient of this substance to last them through the rest of their suckling period. In view of the experiments of Evans [1928], in which he found that the onset of muscular weakness in the young was prevented if they were dosed with a source of vitamin E before they were 15 days old, it can safely be presumed that this missing factor in the milk is the vitamin, and the foregoing experiments suggest that the young of these deficient rats are normal at birth since they can be reared by a normal mother receiving vitamin E in her diet. The subsequent history of these and other fostered rats is being followed and will be the subject of a future publication.

In all the experiments the mother was fed on a vitamin E-free diet throughout but was dosed with just sufficient vitamin E on mating to enable her to produce a living litter. During the gestation and lactation periods the animal received no further dose of the vitamin. In one experiment the animal had been given a larger dose of vitamin E on mating, yet was unable to rear her own litter, 5 of which were born dead, one of which died soon after and the remaining 3 of which were weaklings and died when 16 days old. At this stage the mother evidently had sufficient vitamin E in her milk to fulfil the requirements of normal healthy sucklings for she was able to rear two stock young which she fostered, but this evidently exhausted her store of vitamin E as she failed to rear a further 3 given 8 days later.

From this it may be inferred that vitamin E is not stored for any length of time. In this case the dose given was large but the animal had exhausted her supply by the time she was feeding the third litter. If, however, the dose given is large enough, the animal stores sufficient vitamin E to enable her to complete her pregnancy and to feed and rear her litter. This was shown by an experiment in which two normal stock rats, which had been fed from weaning on an adequate diet containing plenty of vitamin E, were mated and from the day of mating fed on the vitamin E-free diet: both rats had normal gestations, one had a litter of 10 and the other 11. Both fed and reared their litters so that they must have stored sufficient vitamin to carry them through this period. The experiments previously described showed that at birth a rat, whether born of a normal or of a vitamin E-deficient mother, has no store of vitamin E, or so little that it is insufficient to carry it through the suckling period. If this is the case then the abnormality which becomes obvious at about 18 days after birth must be due to vitamin E deficiency and should, if taken in the early stages, be curable by this vitamin. To test this point a sterile rat was mated and given a dose of vitamin E concentrate and had a litter of 6, one of which died. Four of the remaining 5 were given small doses of an active oil from the 6th day onwards. On the 16th day the negative control was weak and the hind feet were contracted while the dosed animals were normal. The negative control became progressively worse; its front feet were affected on the 17th day and on the 21st day it was in a very weak condition, was unable to move and had lost weight. At this stage it was given 0·06 ml. of an oil active in the adult rat in a dose of 0·8 ml.; the following day its condition was worse. The dose was repeated on the 23rd day and on the 24th day there was a marked improvement. On the 25th day the animal was moving normally. No further dose was given and the rat was put on the vitamin E-free diet and died 4 days later. The animals which were dosed from the 6th day onwards remained normal throughout. The experiment was repeated with another litter, but in this case the negative control was dosed 2 days after the onset of the leg weakness and it made a complete recovery.

In another experiment a litter of 9 was born to a vitamin E-deficient mother and was left untreated until 3 days after the onset of the symptoms. At this stage

6 of the litter were alive and all were badly affected, showing both fore and hind leg weakness. All 6 were given several doses of 0·06 ml. of a vitamin E concentrate of which the adult rat dose was 0·31 ml. The details of the experiment are given in Table II.

Table II. *Progress of abnormal young rats dosed with vitamin E concentrate*

Days after birth	No. of animals	Wt. (g.)	Dose (ml.)	Condition
21	6	28–32	0·04	All badly affected, legs weak, feet contracted, hind legs dragging
22	6	—	0·04	Unchanged
23	6	—	0·04	,,
24	6	—	0·06	5 showed improvement, 1 recovered
26	6	26–36	0·06	1 still very bad, 2 practically recovered, 1 improved but feet still contracted, 2 normal
27	6	26–43	0·06	2 unchanged, 1 still weak in left hind foot, 3 normal
28	4	28–45	—	Normal, put on vitamin E-free diet
	2	—	0·06	Still slightly weak
29	2	31 and 50	0·06	Unchanged
30	2	33 and 52	0·06	Slight improvement
31	2	—	0·06	,,
33	2	46 and 60	—	Normal, though left hind foot of one still contracted
34	2	—	—	Both put on vitamin E-free diet, the affected animal gradually regained use of left hind foot

This result was repeated and confirmed with a litter of 6 and again with the 5 survivors of a litter of 7 of which 4 recovered within 10 days and the 5th died on the day following the first dose.

DISCUSSION

Both Evans [1928] and Morelle [1931] described a paralysis in the suckling young of rats deprived of vitamin E. They found that the paralysis affected the hind but never the fore limbs. The mortality was approximately 30%. Evans found that the condition was preventable if the young were treated with a vitamin E concentrate before they were 15 days old. Morelle found that it was prevented by the addition of vitamin E to the mother's diet up to 10 days after mating. Evans made no histological examination, but Morelle examined the nervous system and could find no degeneration. The animals described by Barrie [1937, 1] were obviously comparable, although the condition was much more severe and affected both fore and hind legs and caused carpopedal spasm and convulsions in the final stages. The mortality among these animals was 100%. As in the animals described by Morelle, no nervous degeneration was found, but there was thyroid hypoplasia and involvement of the anterior pituitary.

The present series of experiments, together with the work of Evans and Morelle, shows quite clearly that this paralysis is due to deficiency of vitamin E. The young are apparently normal at birth and under normal conditions would obtain the amount of vitamin E they require from the mother's milk, but when the mother is deprived of the vitamin she does not secrete it in the milk, and unless the young are dosed with vitamin E they suffer from its deficiency and paralysis ensues. This paralysis cannot be caused by any severe lesion as it is fairly readily cured by administration of a large dose of the vitamin. It is

reasonable to suppose that the mode of action of vitamin E is the same in the young as in the adult rat, in which case this paralysis is very probably connected in some way with failure of the function of the anterior pituitary.

It has been known for some time [Evans, 1924; Sure, 1926; Barrie, 1937, 2] that deprivation of vitamin E may cause total or partial failure of lactation, but this failure cannot be due to total lack of milk since the young in the present experiments have all been fat and those that have died have been examined and their stomachs have been found to be full of milk. It may therefore be concluded that although vitamin E in the mother's diet may influence the amount of milk secreted, even if the total amount of milk is adequate still more of the vitamin must be added to the diet before the amount secreted in the milk will be adequate for the requirements of the young.

A very interesting experiment in this connexion was performed by Müller [1936], who found that if sterile vitamin E-deficient rats were fed on human milk the sterility could be cured if the mothers from whom the milk was obtained had been fed on a diet containing vitamin E. This shows that the secretion of vitamin E in human milk is governed by the same factors as in the rat, and although a large volume of clinical evidence for the efficacy of vitamin E in the treatment of habitual abortion is now accumulating, it may well be that this vitamin has a far greater range of clinical application.

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CXCV. THE VITAMIN B₂ COMPLEX

VIII. FURTHER NOTES ON "MONKEY PELLAGRA" AND ITS CURE WITH NICOTINIC ACID

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At present three diseases in experimental animals are known which are analogous with human pellagra. These are canine blacktongue [Goldberger *et al.* 1928], "monkey pellagra" (described in Part VII of this series [Harris, 1937, 1]) and pellagra in pigs [Birch *et al.* 1937]. The discovery by Elvehjem *et al.* [1937; 1938] that nicotinic acid (or amide) cured blacktongue made it desirable that confirmatory experiments should be carried out on the other species. Since tests on "monkey pellagra" were being continued in this laboratory at the time when the preliminary note of Elvehjem and his collaborators appeared, the effect of nicotinic acid was examined without delay. As is now well known, nicotinic acid has in fact been found effective in curing pellagra in pigs [Chick *et al.* 1938], and in human beings [e.g. Fouts *et al.* 1937; Smith *et al.* 1937; Spies *et al.* 1938]. So far as monkeys are concerned only a brief preliminary note about the observations with nicotinic acid has as yet appeared [Harris, 1937, 2]; the fuller experimental details are therefore set out in the present communication.

EXPERIMENTAL

Animals used. The monkeys used (see Fig. 1) varied considerably in size and age, several of them being young animals still rapidly growing, others being nearly full size. Two among the larger ones had been in continuous use since October 1935, first in the experiments reported in the earlier paper and then in later work undertaken in the interim. One of these two, no. 3, had been formerly a positive control receiving yeast (Fig. 1 in Part VII); the other, no. 6, had received in turn numerous curative supplements each followed by a period on the deficient diet (Fig. 4 in Part VII).

Basal diet. This was the same as that described in Part VII and consisted of a modification of Goldberger's blacktongue diet, supplemented with "radiostoleum" (for vitamins A and D) and orange juice (for vitamin C and possibly other factors needed by monkeys). This basal diet has more recently been given baked in the form of a cake. In the present work extra vitamin B₁ (10 I.U. daily per kg. body weight) and lactoflavin (60γ daily) were also provided, as an additional safeguard against the possibility of partial deficiencies of these factors.

Doses of nicotinic acid tested. Various graded allowances of nicotinic acid were tested at intervals, including 30 mg., 15 mg., 10 mg., 5 mg. and 2 mg. per day. The corresponding responses are best judged from the weight curves (Fig. 1).

Symptoms of deficiency. As was shown in the previous paper, monkeys kept on such a basal diet throve provided that a supplement known to contain the P.-P. factor were allowed (e.g. Eli Lilly "343" liver extract powder, or yeast), or if whole wheat were substituted for the maize. Without such supplements the

animals fell ill with the characteristic symptoms of "monkey pellagra"—including loss of appetite, diarrhoea, vomiting, emaciation—and lost weight rapidly and died. No severe skin lesions were noted, but there was often considerable denuding of the fur. The same symptoms were seen again in the present work in those animals receiving no nicotinic acid, or during those periods when it had been withheld for a time. We have also in the later work been specially struck by the anaemic appearance of the animals, the skin becoming very pale in contrast to its normal pink to bluish appearance. Another symptom was the obvious nervousness or "jumpiness" of the deficient animals. Any detailed account of the pathology of the avitaminosis as seen in monkeys is, however, best deferred to a later paper, and for present purposes we may be content to judge the effectiveness of the nicotinic acid by the weight charts. The recovery of good spirits and of normal alertness, as well as the improvement in appearance and loss of symptoms brought about whenever a source of the anti-pellagra factor was given, were always found to be marked by a corresponding gain in the weight curves.

Results with nicotinic acid. Without exception, the administration of nicotinic acid, in doses of between 10 and 30 mg. per day, was followed by a dramatic cure. Even animals which seemed severely ill as a result of the deficiency quickly responded. Appetites were restored and growth was resumed with no apparent lag.
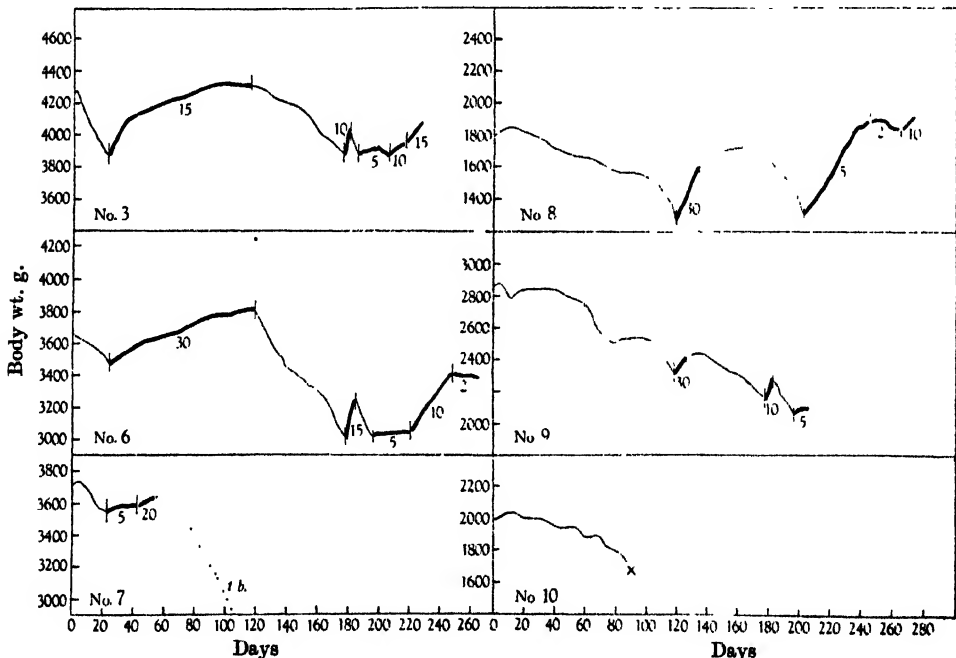
Fig. 1. Growth curves of monkeys. ——— Pellagra-producing diet alone. ▬▬ Supplemented with nicotinic acid. The numbers under the curve refer to the dose of nicotinic acid given in mg. per day.

(The dotted line shows the rapid loss in weight of a monkey which on autopsy was found to have been suffering from tuberculosis.)

The effect on the weight curve and on the liveliness of the monkey was generally already clearly marked on the next occasion when it was weighed, sometimes as soon as 2–3 days after the start of the dosing.

Minimum curative dose. Judging from Fig. 1 a daily dose of about 2 mg. of nicotinic acid is not quite sufficient for rapid recovery in an animal weighing about 2 kg., but doses of 5 mg. and upward are adequate (animal no. 8, cf. also no. 9). For larger monkeys weighing about 4 kg., doses of 5 mg. seem barely enough, but doses of 10 mg., 15 mg. or 30 mg. suffice (monkeys nos. 3, 6 and 7).

These doses may be compared with those required by other species. Young pigs weighing on the average 30–50 lb. have been protected with doses of 25–60 mg. per day [Chick *et al.* 1938], and human beings have been cured with doses of between 60 and 1000 mg. per day [references as on p. 1479]. The minimum requirement for a dog is thought to be about 0·5–1·5 mg. per kg. per day [Elvehjem *et al.* 1938].

Experiments with nicotinamide and β-aminopyridine [cf. Subbarow *et al.* 1938] are still in progress.

SUMMARY

In tests which have been continued for a period of over eight months nicotinic acid has been found consistently effective in curing "monkey pellagra". The curative dose is of the order of 5 mg. for a monkey weighing about 2 kg., or 10 mg. for a monkey weighing 4 kg.

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CXCVI. CHEMICAL NATURE OF THE ULTRA-MICROSCOPIC PARTICLES OF SERUM

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McDonagh [1927] first investigated chemically the fine particles in active Brownian movement which are visible in serum under dark-ground illumination; he termed them "protein particles", and drew attention to their behaviour in an electric current and the modifications in their number during various diseases and recovery therefrom. Peters [1936] confirmed these observations, but did not agree with McDonagh's conception that they were protein, on the ground that normal serum proteins, when fully hydrated, should have a refractive index so near to that of water that the particles would not be visible under the ultra-microscope. He further showed that dehydration and precipitation of the serum proteins with alcohol caused an enormous increase in the number of particles visible. Some lecithin and cholesterol determinations in connexion with this work showed little correlation with the number of particles, yet nevertheless on the evidence then available he favoured the hypothesis that the particles were lipin rather than protein.

More recently, Frazer & Stewart [1937] have published the results of a series of investigations on the effects of feeding on the particle content of the serum of human subjects. They found that the particle content of the serum exhibited a considerable increase following ingestion of fat, but was apparently unaffected by protein or carbohydrate meals, and on this physiological evidence concluded that the particles were lipin in nature, although probably enclosed in an adsorbed protein film.

The present experiments were undertaken in order to investigate the nature of the particles by purely chemical and physico-chemical means, which appeared to be a more direct method of approach than physiological methods, and with a view to finding a chemical explanation of the variations in disease noted by McDonagh and Peters as indicated above.

EXPERIMENTAL

The serum employed was pig serum, which is comparable with human serum as regards particle content, and which could be obtained easily from the slaughter-house on the private farm of this hospital. The serum after separation was preserved with 0·5 % NaF, which was found not to affect the particle content.

The microscope was a dark-ground condensing instrument of the ordinary pattern, but the stage was not fitted with a counting-square. Consequently it was not easy to take a direct count of the numerous and rapidly moving particles, and estimations of the particle content were based on observation of the average apparent distance (in mm.) between neighbouring particles as seen through the eyepiece and compared with the scale on the travelling stage.

Frazer & Stewart [1937] had noticed two distinct types of particles, appearing brightly and dully illuminated respectively. In our experiments we have confirmed and extended this observation, distinguishing three types, viz.: large bright ("LB"), medium dull ("M"), and scarcely visible small dull ("S") particles, which are indicated as such in our results; where more than one type is observed the more numerous of the two appears first, whilst the presence of all three with none predominating is indicated by "(mixed)".

In our tables, apparent distances between particles of 1 mm. or less are indicated by "+ + +" (occasionally "+ + +" is used to show a difference between two results of this order), 1–3 mm. by "+ +", 3–5 mm. by "+", and distances greater than 5 mm. by "±". The "LB" particles, which often showed variations not common to the three types, were on such occasions estimated separately; the above system applies there with the distances increased roughly five times.

Fresh untreated serum generally showed a high content of particles of all sizes. The particle content was unaltered by dilution with 0·9 % NaCl up to three times the original volume, or by prolonged centrifuging in a laboratory (Martin) centrifuge. Table I shows typical examples of sera, and also the effect of dilution. Two slides were usually made of each specimen, sometimes even more.

Table I. *Particles in normal serum. Effect of dilution*

Selection of typical sera from different experiments.

Serum no.	Content	Size of particles	LB
1	+ +	M	
2	– –	S—M	
3	+ + +	(Mixed)	+
4	– –	(Mixed)	– + –
5 (undiluted)	· ·	(Mixed)	
5 (diluted with ½ vol. 0·9 % NaCl)	+ +	(Mixed)	
5 (diluted with 1 vol. 0·9 % NaCl)	– –	(Mixed)	
5 (diluted with 2 vol. 0·9 % NaCl)	· +	(Mixed)	

Precipitation with ammonium sulphate

Serum was half-saturated with ammonium sulphate and the mixture centrifuged. The globulin content of the serum was low, and very little or no precipitate was produced. The amount of ammonium sulphate was later increased to 60 and 70 % saturation. Table II shows a typical result.

Table II. *Serum particles and precipitation with ammonium sulphate*

% Saturation	Before centrifuging		After centrifuging	
	Content	Size of particles	Content	Size of particles
(Original serum)	+ +	M	+ +	M
50	+ +	M	+ +	LB—M
60	+	LB—M	+	LB—M
70	+ +	M	+	M

Some of the uncentrifuged mixtures showed aggregates of globulin.

It is evident from these results that incomplete saturation of serum with ammonium sulphate does not remove the particles from suspension. Adjustment of the serum to pH 4·2 (at which point, as will be shown later, the number of particles shows an apparent maximum) had no effect on their precipitability. Prolonged centrifuging was also ineffective.

The small precipitates produced by incomplete saturation with ammonium sulphate were examined suspended in a small volume of 0·9 % NaCl. A few particles were generally observed, but settled rapidly on the slide. Organic solvents failed to dissolve any of the solid material.

Complete saturation of the serum with ammonium sulphate precipitated the proteins entirely; the supernatant liquid obtained by centrifuging these off proved to be optically empty, showing that the particles were removed together with the albumins. Portions of the dried precipitate, extracted with ether or chloroform, failed to yield a detectable quantity of cholesterol.

Sodium chloride, substituted for ammonium sulphate in a few experiments, produced results similar to the above.

Effect of pH on the particle content

Portions of 10 ml. of serum from the same source were adjusted to pH values over the whole range from 3·0 to 10·0 with the aid of a capillator. The range 3·0–5·0 was examined in detail, since serum proteins are precipitated by acids. Table III shows typical results. The salt concentration was maintained constant by the use of a N solution of HCl in 0·9 % NaCl. The serum mixtures were examined before and after centrifuging.

Table III. *Variation of the particle content with* pH

pH (Original serum)	Content before centrifuging	Content after centrifuging	pH (Original serum)	Content before centrifuging	Content after centrifuging
	+ +	+ +	5·0	┬ +	┤ ┤
3·0	+ +	┤ +	6·0	┄ +	+ ┼
3·2	+ +	+ +	7·0	┄ +	+ +
3·4	┼ + +	+ +	7·6	┄ +	┬
3·6	+ + +	┤ +	8·0	┬ +	┾
3·8	+ +	+ + ┴	9·0	+	┼
4·0	+ +	+ + +	9·2	+	+
4·2	+ +	┬ + +	9·4	±	∴
4·4	+ + +	+ + +	9·6	┴	⊥
4·6	+ +	+ +	10·0	±	−
4·8	+ +	+ +	11·0	−	−

Particle sizes at all pH values (Mixed).

It is seen from these results that the particle content shows a small maximum at pH 4·2–4·4, and on the alkaline side it falls steadily to negligible values at pH 10·0–11·0. Centrifuging at any pH removes very few of the particles if any.

Serum was next incubated at the point of maximum precipitation (pH 4·2–4·4) for 12 hr. at 37°, in an attempt to induce the particles to aggregate or precipitate. The serum, however, set to a stable opaque gel, which when spread on a slide and examined under the ultramicroscope showed large gelatinous aggregates, interspersed with channels containing very small particles in very rapid motion, and in so great a concentration as to cause a hazy appearance from excessive reflexion. This gelation was reversed by restoration of pH to the normal value for serum, and the particle content also returned to normal; it was obviously a protein effect.

A set of tubes of centrifuged serum was then prepared, adjusted to pH values ranging from 3·0 to 4·6, with successive differences of 0·2 unit, and incubated for 24 hr., with examination at intervals for gelation. Table IV shows the results.

These results indicate that gelation occurs most rapidly at pH 3·6, which from Table III is a somewhat lower value than the point of maximum particle

Table IV. *Gelation of serum on incubation at varying* pH

Hours	pH								
	3·0	3·2	3·4	3·6	3·8	4·0	4·2	4·4	4·6
3	0	0	0	0	0	0	0	0	0
5½	0	0	0	TV	0	0	0	0	0
9	0	TV	G	G	AG	0	0	0	0
12	AG	AG	G	G	G	TV	0	0	0
24	AG	AG	G	G	G	TV	0	0	0

("G" = gelified, "AG" = almost gelified, "TV" = turbid and viscous.)

content. This does not suggest that the two effects are associated with each other; the gelation probably differs from the particle effect in being controlled by temperature as well as *p*H.

Behaviour of the particles in a high-speed centrifuge

We are very grateful to Prof. T. B. Davie and Dr J. D. A. Gray of the Department of Preventive Medicine, University of Bristol, for permitting us to make use of their "Ecco-Ultima" centrifuge in an endeavour to effect a separation of the particles from serum.

This machine was capable of a maximum speed of 15,000 r.p.m. Glass tubes were used for the preliminary experiments at medium speed, but they proved to be unable to withstand the highest speeds, being splintered under the strain. A set of duralumin tubes, supplied to fit the machine by Messrs A. Gallenkamp and Co., was found to withstand the strain of the maximum speed. The inner surfaces of the tubes on prolonged use became coated with a film of aluminium hydroxide, but this did not affect the results, and when rubbed up with 0·9 % NaCl gave an appearance under the ultramicroscope that was distinct from that of serum, the suspended particles settling rapidly.

The serum, previously centrifuged at low speed to remove suspended solid material, was usually centrifuged in the high-speed machine for a total period of 1 hr., but this was divided into three periods of 20 min. each, with 15-min. breaks to allow the machine to cool (the temperature was raised a few degrees at the high rate in spite of the cooling jacket).

After centrifuging for an hour, a small precipitate was always found at the bottom of the tube. Before pouring off the supernatant liquid, samples were taken from the surface ("top"), and from the middle and near the bottom of the tube by means of capillary pipettes, the corresponding samples from each of several tubes being mixed with one another. The supernatant liquid was then poured off, and generally sampled also after shaking. The precipitate from the tubes was suspended in 0·9 % NaCl for examination. Table V shows the results of a typical experiment.

The readings show that under the high centrifugal force the "LB" particles tend to move to the surface of the liquid, whilst the remaining particles move towards the bottom, where they ultimately pass out of suspension and collect as a precipitate. At the same time the general uniformity of the particle content of the serum shows that this separation is very gradual. The results suggest, however, that it should be possible to separate out the "LB" particles at very high speeds.

It may be mentioned here that many of our samples of serum, which were more fatty than usual, threw out a disc of white semi-solid material on the surface during their preliminary low-speed centrifuging. This when separated

Table V. *Fractions obtained from high-speed centrifuging of serum*

Serum centrifuged for 1 hr. at 15,000 r.p.m. Samples from top, middle and bottom of tube. Two slides made of each sample.

	Content	Size of particles	LB
Original serum	+ + +	(Mixed)	+
Top sample	+ +	(Mixed)	+ +
	+ +	(Mixed)	+ +
Middle sample	+ +	(Mixed)	−
	+ +	S - M	+
Bottom sample	+	M—S	−
	+	M—S	±
Decanted serum	+ +	· (Mixed)	+
	+ +	(Mixed)	±
Precipitate in suspension	+ +	M	−
	+ +	(Mixed)	−

(The particle content of the suspension was increased by half-saturation with ammonium sulphate.)

and examined was found to be very rich in "LB" particles, whilst the bulk of the serum was comparatively poor. This material was extracted from the serum by ether, and all indications suggested that it was fat.

Behaviour of particles with proteolytic enzymes

Serum was digested with commercial preparations of pepsin, papain and pancreatin, and the effect on the particle content was followed. The enzyme preparations employed were the B.P. mixtures, glycerin. pepsin, glycerin. papain, and liq. pancreaticus. The pepsin and papain mixtures were diluted with 0·9 % NaCl before use. Each was adjusted to its optimum pH value. These mixtures when viewed under the ultramicroscope were found to be free of particles, or else to contain a few that settled rapidly on the slide.

The mixtures of serum and enzyme solutions were incubated in plugged sterile test tubes at 37°, and were examined in duplicate daily under the ultramicroscope. It was found that at least 3 days' incubation was required to produce any noticeable change.

Table VI shows the results of a typical experiment. Continuation of the incubation produced no further change. The total particle content was not reduced by any of the enzymes, whilst pancreatin alone caused a reduction in the "LB" particles. The experiments with pepsin presented considerable difficulty, owing to the ready precipitability of the serum proteins on the acid side of neutrality; the use of moderately concentrated solutions of pepsin resulted in turbid solutions after only 1 day's incubation. Conditions were also favourable for bacterial growth, which spoiled a number of experiments; the addition of preservatives was deemed inadvisable lest the physico-chemical state of the system should be excessively disturbed, whilst sterilization by heat was clearly impossible.

As a further experiment, the fractions obtained from one of the high-speed centrifuging experiments described earlier were incubated with pepsin and pancreatin similarly to the untreated serum. The glycerin. pepsin was replaced by a 0·5 % solution of pepsin in N/30 HCl; liq. pancreaticus was used as before. The top and bottom samples, and the precipitate suspended in 0·9 % NaCl, were all tested. Incubation was continued for 3–4 days. Results are shown in Table VII.

Table VI. *Serum and proteolytic enzymes*

Pepsin. Glycerin. pepsin, B.P., diluted 1 in 20 with 0·9% NaCl. Equal volumes of diluted solution and serum. pH 6·0.

Papain. Glycerin. papain, B.P., diluted 1 in 20 with 0·9% NaCl. Equal volumes of diluted solution and serum. pH 7·0.

Serum control mixture. Equal volumes of serum and 0·9% NaCl. Mixtures incubated at 37°.

	Content	Size of particles	LB
Serum control:			
Before incubation	+ +	(Mixed)	+ +
After 3 days' incubation	+ +	(Mixed)	+ +
Serum-pepsin:			
Before incubation	+ +	(Mixed)	+ + +
After 3 days' incubation	+ +	(Mixed)	+ +
Serum-papain:			
Before incubation	+ +	S	+
After 3 days' incubation	+	(Mixed)	+
	+ +	(Mixed)	+ + +

Pancreatin. Liq. pancreaticus, B.P., undiluted. 2 vol. to 1 vol. of serum. pH 8·0.

Serum control mixture. 2 vol. 0·9% NaCl to 1 vol. serum. Mixtures incubated at 37°.

	Content	Size of particles	LB
Serum control:			
Before incubation	+ +	(Mixed)	+
After 3 days' incubation	+ +	(Mixed)	-
Serum-pancreatin:			
Before incubation	- +	(Mixed)	+
After 3 days' incubation	+ +	S— M	-
	+ +	(Mixed) (Settled rapidly)	(+)
Pepsin solution	-		
Papain solution			
Pancreatin solution	-		

Table VII. *High-speed centrifuge and proteolytic enzymes*

Pepsin. 0·5% pepsin in N/30 HCl. Equal volumes of solution and serum. pH 6·0. Mixture incubated at 37°.

	Content	Size of particles	LB
Top sample:			
Before incubation	+	M— S	+
After 4 days' incubation	+ +	(Mixed)	+ +
Bottom sample:			
Before incubation	+	M	-
After 4 days' incubation	+ +	M	-
Precipitate in suspension:			
Before incubation	-	(Particles settled)	
After 4 days' incubation	-		

Pancreatin. Liquor pancreaticus, B.P., undiluted. 2 vol. to 1 vol. of serum. pH 8·0. Mixtures incubated at 37°.

	Content	Size of particles	LB
Top sample:			
Before incubation	+ +	L—M	+ +
	+	(Mixed)	+ +
After 4 days' incubation	+	(Mixed)	+
	+ +	M— S	+
Bottom sample:			
Before incubation	±	M— S	-
After 4 days' incubation	+ +	M —L	-
	+	M— S	-
	+	(Mixed) (Particles settled rapidly)	-
Precipitate in suspension:			
Before incubation	+	M—S	-
After 3 days' incubation	±	S—M	-
Pepsin solution	-		
Pancreatin solution	-		

As was usual in the centrifuging experiments the "LB" particles were concentrated in the top sample. The concentration was noticeably reduced by pancreatin, but apparently unaffected by pepsin. "LB" particles were absent from the other samples, and the small and medium particles in all showed no significant change with either enzyme.

The disappearance of the "LB" particles on digestion with pancreatin is therefore the only noteworthy change caused by proteolytic enzyme preparations. Pancreatin contains lipase as well as trypsin, and its selective action on "LB" particles might well be explained by this fact, which would suggest that the "LB" particles are fat. The absence of any change in the small particle contents on digestion with proteolytic enzymes will be discussed in a later section.

The suspension of the precipitate from the high-speed centrifuge in 0.9% NaCl showed a slight reduction in its total particle content on digestion with pancreatin. This is to be expected on the hypothesis that the smaller particles are protein in nature, since the total amount of protein material present in such a suspension is necessarily limited. The precipitate also showed a reluctance to form a stable suspension in the acid pepsin mixture, the particles settling rapidly: this result when viewed together with the previous observations of precipitation of proteins and increase of particle content with fall of pH favours further the protein hypothesis.

Ultramicroscopic particles and filtration

The effect of filtration through paper and certain adsorbents on serum was studied in comparison with the effect of similar filtration on cow's milk, which when examined under the ultramicroscope shows a high content of particles of all sizes ranging from large fatty globules to particles similar to those observed in serum.

Milk. Milk was first filtered through a folded "Green's $500\frac{1}{2}$" (agar) filter paper, and afterwards through a cone of calcium sulphate-carbonate mixture, prepared by moistening plaster of Paris, and after it had set powdering it in a mortar and adding 5% of precipitated chalk. The adsorbent was supported in an agar paper. Filtration was very slow, but sufficient filtrate was always obtained to permit the examination of all filtrates on the same day.

The filtrate from the agar paper was still white and opaque, but after passing through the $CaSO_4$—$CaCO_3$ mixture it appeared only opalescent.

A second portion of milk, after filtration through the agar paper, was filtered through a similar cone of "Kaylene" (colloidal kaolin). This also yielded an opalescent filtrate. Occasionally with both the adsorbents the filtration required repetition.

The optical examination of the milk and the various filtrates is recorded in Table VIII.

Table VIII. *Filtration of milk through adsorbents*

	Content	Size of particles	
Untreated milk	$+++$	M	Many large globules on slide. Film not uniform
Agar paper filtrate	$+++$	M	No large globules but numerous small globules and submicrons. Film uniform
$CaSO_4$—$CaCO_3$ filtrate	$++$	M	No globules
"Kaylene" filtrate	$++$	M—L	No globules

The particles observed in the milk show a noticeable difference from those in serum, being circular in appearance and steadier in brilliance. The optical appearance of the final milk filtrates was unaffected by centrifuging in the low-speed machine.

The two filtrates from the $CaSO_4$—$CaCO_3$ mixture and "Kaylene" filtrations were acidified with N HCl. A flocculent precipitate was produced that showed no Brownian movement; when this was removed by filtration or centrifuging a clear solution was obtained which was optically empty. The precipitate was insoluble in chloroform or ether, and when dried did not give the Salkowski reaction for cholesterol; it was most probably casein.

Serum. Serum was now subjected to filtration through agar paper and both adsorbents with exactly the same technique. Table IX shows the results obtained with two samples of serum filtered through "Kaylene", and one of them through $CaSO_4$—$CaCO_3$ mixture. Serum "A" was fresh, whilst serum "B" had been in cold storage for a week.

Table IX. *Filtration of serum through adsorbents*

		Unfiltered		Filtered	
Serum	Adsorbent	Content	Size of particles	Content	Size of particles
A	"Kaylene"	+ ?	M	+	(Mixed)
			(3 slides)		(3 slides)
A	$CaSO_4 \cdot CaCO_3$	- +	(Mixed)	+ -	(Mixed)
		- -	S	+ -	(Mixed)
		- +	S—M	+	S—L
B	"Kaylene"	?	(Mixed)	+	S
		- +	(Mixed)	+	M
		+ -	(Mixed)	+	(Mixed)

It will be seen that filtration made very little difference to the particle content. Acidification of the filtrates resulted in a steady precipitation of serum proteins according to the amount of acid added; the precipitates on centrifuging off and drying were found to be free of cholesterol. The supernatant liquids were opalescent and heavily charged with particles.

Serum "B" showed an interesting result. Unlike many of our samples, it had not become contaminated with haemoglobin through haemolysis while separating from its blood clot. It was noticed that the yellow colour in the "Kaylene" filtrate had deepened almost to brown; in spite of this, the particle content showed no increase, but rather an apparent fall. This suggests that the lipochrome pigments of serum have no connexion with the ultramicroscopic particles. In view of the lack of effect of dilution of serum on the particle content, which will be discussed later, we cannot regard this result as absolute evidence of the retention of particles by kaolin. Serum "A", which contained haemoglobin, showed no colour change on filtration.

Lipins. For comparison, concentrated colloidal suspensions of cholesteryl oleate (B.D.H.) in soap solution, and of ovolecithin in distilled water, were filtered through agar paper and then through each adsorbent. The optical appearance of the suspensions before filtration showed distinct differences from that of serum. The cholesteryl oleate suspension showed circular, steadily-brilliant particles similar to those observed in milk, and also the same globules, some of which were small enough to pass through the agar paper. On the other hand, the lecithin particles were much less sharply defined than serum particles. Table X shows the effect of filtration on these suspensions.

Table X. *Filtration of lipin suspensions*

	Ovolecithin		Cholesteryl oleate	
Adsorbent	Content	Size of particles	Content	Size of particles
(Unfiltered suspension)	+ + +	M—S	+ +	(Mixed)
"Kaylene"	+ + +	S	+	(Mixed)
CaSO₄—CaCO₃	−	−	±	(Mixed)

The ovolecithin was adsorbed completely by the CaSO₄—CaCO₃ mixture, the filtrate being clear and colourless, and showing no Tyndall phenomenon— a direct contrast to the lack of effect on serum. The reduction of particle content in cholesteryl oleate suspensions by filtration through CaSO₄—CaCO₃ mixture was not regular in all our experiments, and the filtrate always showed a positive Tyndall effect.

Serum particles and extraction with organic solvents

The last group of experiments recorded in this paper was an investigation of the effect on the serum particles of extraction with the ordinary fat solvents.

Extraction was carried out by shaking the serum and solvent together in a mechanical shaker for periods of 6 hr.

Chloroform, carbon tetrachloride, benzene and light petroleum all caused denaturation of the serum proteins, generally precipitating them or forming a gel; the aqueous layer, examined when possible, contained particles in very large numbers, whilst the residues from evaporation of the organic solvent layers gave optically empty suspensions in 0·9 % NaCl. Ether was free of this disadvantage.

Serum was extracted three times with five times its volume of ether by the above technique. The aqueous layer after examination was evaporated to dryness and extracted again with ether in a Soxhlet apparatus, this second extract and the residue in the extraction thimble being weighed.

The ether extract from the serum, when evaporated to dryness, yielded an oily, evil-smelling residue, which was weighed and examined suspended in 0·9 % NaCl. The Salkowski test for cholesterol applied to this residue was positive.

The saponification values of the ether extracts of both the serum and the dried serum were determined to give an indication of their fat contents. Results are shown in Table XI.

Table XI. *Exhaustive extraction of serum with ether*

Serum (60 ml.) shaken three times with 5 vol. of ether for 6 hr.

	Content	Size of particles	LB
Original serum	+ + +	(Mixed)	+ + +
Ether-extracted serum	+ +	(Mixed)	+
Ether extract in 0·9 % NaCl	−		

	Weight of extract (mg.)	Saponification value
Ether extract of serum	127·6	130
Ether extract of dried serum	10·0	340

Weight of dried residue, 4·9 g.

It is seen from the table that practically the whole of the ether-soluble material was removed from the serum by the exhaustive treatment with ether. At the same time the "LB" particle content was very considerably reduced, but the total number of particles was not much less than before extraction.

Similar extractions with ether containing 6 and 12 % of alcohol, which according to Mâcheboeuf & Sandor [1932] will remove the lipins completely from horse serum without affecting the proteins, caused considerable denaturation with pig serum, the aqueous layer becoming turbid and heavily charged with particles. Suspensions of the extracts in 0·9 % NaCl were optically empty. The Salkowski reaction was positive, and lecithin also was identified in the extracts by hydrolysing with acid and formation of choline reineckate [Beattie, 1936].

Addition of alcohol to the serum followed rapidly by a large volume of ether also increased the number of particles by denaturing the proteins.

Table XII shows an experiment similar to that of Table XI, carried out on human serum from a case of paratyphoid fever. This serum was comparatively poor in "LB" particles at the outset. Results otherwise resemble those in Table XI.

Table XII. *Extraction of human (paratyphoid) serum with ether*

Serum (10 ml.) shaken three times with 5 vol. of ether for 6 hr.

	Content	Size of particles	LB
Original serum	+ +	M -S	+
Ether-extracted serum	+ +	M—S	±
Ether extract in 0 9 % NaCl	—		

	Weight of serum (mg.)	Saponification value
Ether extract of serum	36·8	160
Ether extract of dried serum	4·6	50

Weight of dried serum, 580·6 mg.

In the next experiment three separate volumes of pig serum were extracted once with the same five volumes of ether to obtain a large quantity of ether-soluble material. The ether was partly taken up into the serum layers during the second and third extractions, forming a viscous mixture which, when allowed to stand in the refrigerator in a separating funnel, separated slowly into three layers, a "middle layer" of colourless, viscous liquid being formed between the usual serum and ether layers. The course of the separation of the ether and "middle layer" was followed in the ultramicroscope.

The ether extract, when evaporated down, yielded a moderately large amount of solid material, whilst Soxhlet extraction of the dried serum and "middle layer" with ether yielded relatively small quantities, that from the dried serum being negligible. The saponification values of all three extracts were determined.

The total content of lipin material of the ether extract of the serum was determined roughly by extracting a weighed quantity of the solid material with light petroleum and weighing the extract. About 90 % of the material was dissolved.

We are indebted to Prof. E. L. Hirst and his staff for kindly carrying out a micro-determination of the nitrogen content of the material.

It will be seen from Table XIII that a large number of "LB" particles were left in the serum mixture after shaking with ether was completed, but that on standing these all passed into the "middle layer". The latter after drying contained a considerable percentage of ether-soluble material, but the dried serum layer was practically free of it. The ether extract of the serum was apparently all fat except for 10 % (about 5 % being protein from the nitrogen figure), yet the total serum particle content was unchanged by extraction with ether. The

Table XIII. *Extraction of serum with restricted quantities of ether*

3 vol. serum, 60 ml. each, extracted for 6 hr. with the same 300 ml. ether

	Content	Size of particles	LB
Original serum	+ +	(Mixed)	+ + +
Ether extract in 0·9 % NaCl	−		
Unseparated serum mixture	+ +	(Mixed)	+ + +
Half-separated serum layer	+ +	(Mixed)	+ +
Completely separated serum layer	+ +	(Mixed)	+
"Middle layer"	+ +	(Mixed)	+ + +

		Ether extracts	
	Serum	Dried "middle layer"	Dried serum layer
Weight of dried material	—	566 mg.	3800 mg.
Weight of extract	242·6 mg.	20·2 mg.	12·2 mg.
% of dried material extracted	—	3·57 %	0·09 %
Saponification value	120	171	160
Iodine value	75	8	6
Nitrogen	0·4 %	.	—
% of material soluble in light petroleum	90 %	—	—

relatively large quantity of ether-soluble material in the "middle layer" as compared with the serum layer, and its high saponification value and low iodine value, suggest that the "LB" particles that passed into the "middle layer" were composed of saturated neutral fat.

The absence of any effect on the number or size of the particles in serum by treating with equal volumes of sodium tauroglycocholate solutions in concentrations up to 10 % showed that the particles were not free fatty acid. They also were not precipitated by ricin, which showed that they were not cholesterol.

Extraction after high-speed centrifuging. Extraction three times with ether of a sample of serum that had been centrifuged for an hour in the "Ecco-Ultima" machine at 15,000 r.p.m. yielded the same amount of fatty material as did extraction of an equal volume of uncentrifuged serum. The precipitate which separated out gave no indication of being soluble in ether, whilst on the other hand, half-saturation with ammonium sulphate of its suspension in 0·9 %

Table XIV. *Extraction of milk filtrates*

"Kaylene" and CaSO₄—CaCO₃ filtrates, 30 ml. Extracted three times for 6 hr. with 150 ml. ether

	Content	Size of particles
"Kaylene" filtrate	+ + +	M
Ether-extracted "Kaylene" filtrate	+ + + +	M
CaSO₄—CaCO₃ filtrate	+ + +	M
Ether-extracted CaSO₄—CaCO₃ filtrate	+ + + +	M

	Weight of material	Saponification value
"Kaylene" filtrate:		
Ether extract	19·0 mg.	301
Ether extract of dried residue	8·4 mg.	—
CaSO₄—CaCO₃ filtrate:		
Ether extract	4·0 mg.	670
Ether extract of dried residue	7·2 mg.	—
Weights of dried residues:		
"Kaylene"	2·3 g.	—
CaSO₄—CaCO₃	2·4 g.	—

NaCl increased considerably its particle content, suggesting a protein origin for the particles.

Milk filtrates. Table XIV shows the figures obtained by extracting the "Kaylene" and $CaSO_4$—$CaCO_3$ filtrates of milk in a similar way to serum. The amount of fat obtained in each case was negligible, but the particle content, already large, showed a small increase.

These results support the view that both milk and serum particles are protein in nature.

Sera from different species

A few comparative experiments were made with pig, sheep and cow sera. Sheep serum resembled pig serum both externally and ultramicroscopically; cow serum, on the other hand, was coloured an intense yellow by lipochrome pigments. The optical pictures (Table XV) show no great differences.

Table XV. *Comparison of sera from different species*

Serum	Content	Size of particles	LB
Pig	+ +	M—S	+
Sheep	+ +	M—S	+
Cow	+	S—M	−

Sheep and cow sera were extracted three times with five volumes of ether as for pig serum. The sheep serum was unaffected by the extraction, but a considerable quantity of the yellow pigment was removed from the cow serum; the concentrations in the unextracted and extracted sera as compared in a Hellige colorimeter were in the ratio 9 : 5. The ether extracts were evaporated and weighed. Table XVI shows the optical results and the weights of the extracts.

Table XVI. *Extraction of sheep and cow sera*

30 ml. each serum, extracted three times for 6 hr. with 150 ml. ether

	Content	Size of particles	LB
Sheep serum	+ +	S—M	+
Ether-extracted sheep serum	+ +	S—M	−
Cow serum	+ +	(Mixed)	+
Ether-extracted cow serum	+ +	(Mixed)	−

Weights of extracts: Sheep serum, 40 mg.   Cow serum, 42·8 mg.

The extract from cow serum was soluble in light petroleum except for about 6·6 %.

These figures show that the behaviour of the particles in sheep and cow sera with ether is essentially the same as that of pig serum particles. The removal of the pigment from cow serum has no visible effect on its particle content.

DISCUSSION

We have indicated after each of our lines of investigation the conclusions we have reached from the results therefrom as to the chemical nature of the ultramicroscopic particles in serum. Certain of these and points arising therefrom require further discussion.

With regard to the smaller particles, practically all the evidence shown supports McDonagh's conclusion that they are protein in nature. They may consist of both globulin and albumin; the results with half-saturation with ammonium sulphate show in fact that both can produce particles. The small maximum shown in the particle content when serum is brought to pH 4·0–4·2

probably represents an isoelectric point for one particlular protein. In connexion with this conclusion as to the smaller particles we are greatly indebted to Dr L. F. Hewitt for the gift of a sample of his "fat-free" horse serum [Hewitt, 1927], which when viewed under the ultramicroscope gave the reading " + + M—S ". The experiments with sera from different species recorded in this paper indicate that there is no essential difference between the small particle contents of any particular species, so that the above reading can be taken as typical for fat-free normal sera.

It is interesting to consider the effects of diluting the serum, which contrary to expectation does not appear to cause any considerable reduction in its small particle content; the particles are obviously being reinforced from some source within the serum. The ether extraction results make it highly improbable that the serum lipins are the source concerned so that the extra particles must therefore be drawn from the serum proteins. This does not at first appear to be an acceptable conclusion, since dilution would be expected to facilitate hydration of the proteins. We must postulate an "equilibrium concentration" of unhydrated protein in every serum, which always tends to re-establish itself on dilution, and possibly also on concentration, of the serum. Such an equilibrium concentration may probably be of considerable physiological and pathological importance. It represents in undiluted normal serum only a very small fraction of the total serum proteins, on which it is not completely dependent. It should explain the apparent lack of effect of the proteolytic enzymes on the particles, since any unhydrated protein that was digested would immediately be replaced by dehydration of fresh protein. The reduction by pancreatin of the particle content of the suspension of the precipitate from high-speed centrifuging is to be expected on this hypothesis, for the suspension contains no reserve of hydrated protein to replace digested particles.

The large bright particles tend to "follow the fat" in every process to which the serum has been subjected, and are most probably neutral fat, which may be saturated or unsaturated.

Frazer & Stewart [1937] reported that the number of particles of all sizes in normal human serum was increased by ingestion of fat, but apparently unaffected by protein. They concluded from these observations that the particles were of lipoid origin. Our results do not support this conclusion, except for the "LB" particles. We suggest that the absorption of the extra fat into the blood causes changes in the colloidal state of the serum, which lead to an increase in the equilibrium concentration already postulated of the unhydrated protein, which recovers its normal value on removal of the fat. Protein passing into the blood does not affect the colloidal picture in this way, since the equilibrium concentration, as stated above is governed principally by physical factors, the absolute concentration of the serum proteins being only indirectly concerned if at all. The equilibrium concentration hypothesis should also explain Frazer & Stewart's observation that the particles in normal serum are maintained at a constant level in starvation.

One of us [Peters, 1936] has recorded the examination of serum from a case of acute tuberculosis. The picture was heavily charged with particles and aggregates of gelatinous material, and was almost identical with that which we obtained with serum gelified by incubation at pH 4·2–4·4. Since serum globulin is known to increase largely in tuberculosis, we conclude from this observation that the clotting of fibrin in tuberculous blood leaves the serum so highly concentrated that the equilibrium concentration effect is overcome, and the globulin is dehydrated in large quantities and gelified.

SUMMARY

1. The ultramicroscopic particles in serum are of three distinct sizes: large bright ("LB"), medium-dull, and small dull.

2. The number of particles is not diminished by dilution up to three times the volume of the serum with 0·9 % NaCl.

3. The particles are not appreciably affected by half-saturation of the serum with ammonium sulphate, although some particles are to be seen when the globulin precipitate so obtained is suspended in 0·9 % NaCl. Complete saturation removes the particles with the proteins.

4. Variation of pH towards the alkaline side causes disappearance of the particles. On the acid side the particles increase with the acidity until the proteins begin to precipitate. A small maximum occurs at pH 4·2-4·4.

5. The particles are vastly increased in number when serum is gelified by incubation at pH 3·6. Restoration of pH to normal also restores to normal the particle content.

6. When serum is centrifuged at 15,000 r.p.m., the "LB" particles move to the surface of the liquid, but the smaller particles appear to be drawn to the bottom.

7. The smaller particles in serum are apparently unaffected by proteolytic enzymes, but the "LB" particles are removed by pancreatin, probably through the action of lipase.

8. Filtration of milk through colloidal kaolin or $CaSO_4$—$CaCO_3$ mixture removes all the fat, but leaves a suspension of ultramicroscopic particles resembling those of serum. Serum particles are scarcely affected by filtration through either adsorbent. Lipins are either partly or wholly removed from suspension by $CaSO_4$—$CaCO_3$ mixture.

9. Prolonged extraction of serum with several changes of ether removes all the fat, and the "LB" particles disappear at the same time, but the number of the smaller particles is unaffected. Extraction of several charges of serum with the same ether results in formation of a "middle layer" which contains some saturated neutral fat, and also a large number of "LB" particles.

10. The sera of pigs, sheep and cows, and human serum from a case of paratyphoid fever, show no essential difference in their particle contents nor their behaviour with ether. The lipochrome pigments in cow serum are extracted by ether without affecting the particle content.

11. It is concluded that the smaller particles are protein as originally stated by McDonagh [1927], but that the "LB" particles are neutral fat.

12. An "equilibrium concentration" of unhydrated protein in serum is postulated, which is governed partly or wholly by physical factors and tends to re-establish itself on dilution and possibly concentration of the serum.

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CXCVII. THE VITAMIN A₁ AND A₂ CONTENTS OF MAMMALIAN AND OTHER ANIMAL LIVERS

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THE liver fats of animals in general rank amongst the richest sources of the growth-promoting and anti-xerophthalmic substance known as vitamin A which, in addition to its biological activity, is characterized by an ultra-violet absorption band near $328 m\mu$ and by the blue colour that it gives with antimony trichloride in chloroform (main absorption maximum at $620 m\mu$). These properties of the vitamin are shown with slight modification by the liver fats both of marine fish and of mammals. When, however, the first preparations of really potent concentrates of the vitamin were obtained [Karrer *et al.* 1931; Heilbron *et al.* 1932] they were obviously heterogeneous, notably because they contained small but variable quantities of a substance exhibiting an absorption band at $693 m\mu$ in the SbCl₃ blue colour [Heilbron *et al.* 1932]. It has since been observed that the liver fats of certain Russian freshwater fish contain more of the $693 m\mu$ chromogen than of the more common one characterized by a $620 m\mu$ band [Lederer & Rosanova, 1937] and more detailed spectrophotometric examinations of the liver oils of a variety of freshwater fish [Lederer *et al.* 1937; Edisbury *et al.* 1937; Gillam *et al.* 1938; Edisbury *et al.* 1938] have confirmed this. The same investigations have also shown that the ultra-violet absorption spectra of the oils are characterized by two maxima near 280 and $350 m\mu$, respectively, instead of the single maximum at $328 m\mu$ common to the liver oils of marine fish.

It has been suggested that the $693 m\mu$ chromogen might be a second vitamin A, possibly specific to freshwater fish [Lederer *et al.* 1937], whilst Edisbury *et al.* have tentatively named it vitamin A₂ [1937] and later "factor A₂" [1938]. Although in our earlier concentration of the substance it was not found possible to separate it completely from vitamin A₁ the results of feeding experiments on rats [Gillam *et al.* 1938] indicate that it actually possesses the biological activity always associated with vitamin A₁, whilst the chemical evidence suggests that it is probably a higher isologue ($C_{22}H_{32}O$) of this vitamin which, itself, has been shown to have the formula $C_{20}H_{30}O$ [Karrer *et al.* 1931; Heilbron *et al.* 1932]. More recent experiments with the physiologically potent concentrate previously examined have shown that it contains another chromogen (645–$650 m\mu$) the presence of which makes the $620 m\mu$ chromogen value too high [Lederer, 1938]. On the assumption that this chromogen has no vitamin A activity itself, Lederer [1938] draws the conclusion that more of the total activity of the concentrate must be due to the $693 m\mu$ chromogen than was previously supposed. This further justifies the title vitamin A₂, as also does the fact found by Wald [1937, 1, 2] that the $693 m\mu$ chromogen can replace vitamin A₁ in the visual cycle that occurs in the retinae of freshwater fish.

The generalization that is beginning to emerge from these results is that whereas marine fish liver oils contain the $620 m\mu$ chromogen (vitamin A or A₁) with only traces of the $693 m\mu$ chromogen (vitamin A₂),[1] freshwater fish liver oils

tend to contain more vitamin A_2 than vitamin A_1, or at least a larger proportion of vitamin A_2 than do the marine fish liver oils. The only mammals that have been examined for vitamin A_2, namely, the rabbit [Edisbury *et al.* 1938] and the whale (private communication from Dr R. A. Morton), apparently contain none of this substance.

The present paper records the results obtained by the examination of the livers of a series of animals and birds with particular reference to the total vitamin A content of the liver and the presence or absence of vitamin A_2.

EXPERIMENTAL

The procedure adopted in all cases was, firstly, hot saponification of the fresh liver with KOH (2 ml. 10 % KOH in 50 % aqueous alcohol per g. of fresh liver: time of saponification 1–3 hr.) until the tissue was completely broken down. After cooling, the mixture was diluted with water and extracted with ether, the resulting extract being washed with water, dried over anhydrous Na_2SO_4 and evaporated to dryness in N_2. Part of the unsaponifiable matter so obtained was treated with $SbCl_3$ in chloroform, the blue colour being examined spectroscopically by methods already described [Heilbron *et al.* 1931]. Values for the relative amounts of vitamins A_1 and A_2 present were obtained from the ratio of the intensities of the absorption bands at 620 and 693 mμ, respectively, of the blue solution. The remaining unsaponifiable matter in chloroform was diluted 10–20 times with alcohol and its ultra-violet absorption spectrum determined photographically, using a Hilger E 3 quartz spectrograph. The vitamin A content was taken as the mean of the two values obtained from the intensity of absorption of the alcoholic and chloroformic $SbCl_3$ solutions, respectively, on the provisional basis for pure vitamin A of $E\,|\,^{1\,°/_o\,620\,m\mu\,=\,5000\,(SbCl_3)}_{1\,cm\,\,328\,m\mu\,=\,1600\,(alcohol)}$ [Carr & Jewell, 1933].

The results are recorded in Table I.

DISCUSSION

Consideration of the data and of previous work [cf. Kuhn, 1933] leads to the conclusion that in marine fish in general (or possibly in some of the animals on which they feed) carotene is ingested (either directly or indirectly from the phytoplankton) and the $C_{40}H_{56}$ molecule is broken down into C_{20} fragments constituting the typical vitamin A. The small amount of vitamin A_2 normally present would appear either to be produced as a by-product of this process in the fish themselves, or to be received by them preformed to this small extent from the lower animals on which they feed. On the other hand, freshwater fish (or again some lower organisms eaten by them) must be able, in addition, to break down the carotene molecule into C_{22} and C_{18} fragments, the C_{22} fragment, at least, appearing in the liver in greater concentration than is normally met with in marine fish livers.

The complete absence of vitamin A_2 from the livers of the majority of both the individuals and the species of mammals and birds examined indicates that

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[1] Karrer has suggested the name "Axerophthol" for the classical vitamin A and the class name "Axerophthols" for the higher isologues [Euler *et al.* 1938]. Using Karrer's nomenclature, the particular axerophthol ($C_{22}H_{31}O$) which our earlier experiments indicate as the 693 mμ chromogen would be called β-*apo*-5-carotinol. Karrer and his collaborators have prepared what they believe to be this compound and whilst it gives a blue colour with $SbCl_3$ the position of the absorption band that gives rise to the colour is apparently quite different from 693 mμ. If the formula of the compound is beyond doubt then the formula suggested for the 693 mμ chromogen will need modification.

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Table I

Animal	Source, etc.	Wt. fresh liver g.	Estimated total vitamin A mg.	Vitamin A mg. per 100 g. liver	693 mμ chromogen	Ratio of intensities 693/620 mμ
Lion *Felis leo*	Zoological Gardens (20 yr. old)	2000	160	8·0	Absent	0·057
Gibbon *Hylobates concolor*	Zoo (6 months old)	110	0·75	0·70	,,	Not measurable
Giant monitor *Varanus salvator*	Zoo	(a) 200 (b) 113	500 74	250 65	Present —	0·11 —
Python *Python reticulatus*	Zoo (very old animal)	800	688	86	Absent	0·023
Alligator *Alligator mississippiensis*		225	8·0	3·5	,,	<0·20
Rabbit *Lepus cuniculus*	Wild	27·6	4·75	17·2	,,	0·073
Rat *Mus decumanus*	Wild town animal, ♂	9·2	0·55	6·0	,,	0·031
Mouse *Mus musculus*	Laboratory animals (a) Av. of 9 (b) Av. of 15	1·63 1·14	0·013 0·0125	1·1 1·1	,, ,,	Not measurable —
Guinea-pig *Cavia porcellus*	Laboratory animals Av. of 3	10·4	0·07	0·68	,,	0·076
Dog		278	3·06	1·1	,,	<0·20
Cat		66·5	2·4	3·6	,,	0·033
Ox		5235	340	6·5	,,	0·052
Cow		6075	132	2·17	,,	0·082
Lamb		(a) 506 (b) 280	56 51	11·0 14·7	,, ,,	0·058 0·063
Pig		(a) 844 (b) 1000	67 10	7·94 1·0	,, ,,	0·044 —
Frog *Rana temporaria*	Local, av. of 6	0·41	Order of 0·0043	1·04	,,	Not significant
White Whale *Beluga leucas*		—	—	—	,,	0·066
Hen	Av. of 2	28	3·4	12·1	,,	0·068
Duck	Av. of 4	24	2·93	12·2	,,	<0·14
Turkey		(a) 118 (b) 128	24·7 26·4	21·0 20·7	,, ,,	0·044 0·06
Pigeon	Av. of two young birds	4·2	1·37	32·5	,,	0·10
Kingfisher *Alcedo ispida*	Cheshire (one young fledgeling: found dead)	1·9	0·15	7·7	Not detected	—
Seal *Phoca vitulina*	Russia	—	—	—	Present	0·20
Otter *Lutra vulgaris*	Cumberland	191	90	47	,,	0·22
Rat (fed on vitamin A₂ concentrate)		—	—	—	,,	1·40

they cannot convert carotene directly into vitamin A_2 for, in the herbivorous animals at least, there can be no doubt that the diet normally contains relatively large quantities of carotene. The question arises, however, whether mammals can actually pass preformed vitamin A_2 into the liver when it is taken into the digestive tract. To test this point Dr Lederer very kindly supplied us with extracts of the livers of rats fed on concentrates of vitamin A_2. Examination of these extracts (cf. Table I) revealed the presence of relatively large quantities of the 693 mμ chromogen; in fact the livers of the rats were, chromogenically, very similar to those of freshwater fish.

Since mammals normally do not appear to manufacture the 693 mμ chromogen but can pass it into the liver when it is contained in the food eaten, it was argued that mammals that feed on fish should store some of this substance in their livers. Interesting test cases of this point are provided by the seal, for a liver extract of which we are indebted to Dr Lederer, and the otter, of the liver of which a sample was obtained from Cumberland through the kindness of Dr W. S. M. Grieve. Examination showed that both these livers contained vitamin A₂ in easily detectable amounts (cf. Table I). On the other hand the only specimen of a bird feeding on freshwater fish that we could obtain (i.e. the kingfisher) was found to have no detectable vitamin A₂ in its liver[1] (cf. Table I). Thus of all the mammals examined the only ones having the 693 mμ chromogen present in the liver were those known to feed on, or to have fed upon, fish. Of the remaining animals and birds examined the only other having any detectable quantity of this chromogen was the giant monitor in which, however, the proportion of this substance present was very small (cf. Table I).

It is interesting to note that the python and the giant monitor had the largest total weight of vitamin A₁ per liver of all the species examined: moreover, on the basis of percentage weight of vitamin in the liver, these two species stand alone. The particular python examined was a very old 25 ft. specimen and it may be that, as in the case of the halibut where the vitamin content of the liver increases with age, individuals of this species also accumulate vitamin A as they become older. Of the remaining species examined with the exception of the otter the percentage of vitamin A in the liver only varied within relatively narrow limits; thus in 18 species the average was 8·3 mg. per 100 g. fresh liver, whilst the limits of variation were of the order 1 to 32 mg. per 100 g. When mammals are compared with fishes much larger variations are, however, observed [cf. Edisbury *et al.* 1938].

In this preliminary survey only a few individuals of each species have been examined but it is probable that if more were examined much wider variations would be found. On the other hand the work of Davies & Moore [1935] indicates that the level of vitamin A in the liver of the rat usually falls within narrow limits; these workers have shown that when rats are fed with very high dosages of vitamin A the abnormally high resultant value for the vitamin content of the liver rapidly falls to normal as soon as the vitamin intake is reduced.

The differences between freshwater fish, marine fish, mammals and birds in the reaction of their liver extracts towards SbCl₃ are summarized in Table II.

Table II. *Summarized data on the relative amounts of 693 and 620 mμ chromogens in the livers of fish, animals and birds*

	No. of species included*	Ratio of the intensities of absorption at 693/620 mμ		693 mμ absorption band
		Range found	Average	
Freshwater fish†	11	0·25–2·62	1·680	Usually strongly positive
Marine fish†	11	0·04–0·17	0·110	Normally just detectable
Animals (almost all mammals)	11	0·03–0·17	0·060‡	Only rarely present
Birds	5	0·04–0·10	0·068‡	Only rarely present

* Some of these species have been represented by many specimens, others by only one.

† Cf. Gillam *et al.* [1938] and Lederer [1938] for some detailed examples.

‡ These values, which might be expected to be zero in the absence of the 693 mμ band, probably represent the overlap of the 693 mμ band at 620 mμ.

[1] The author is indebted to Miss Legge of the Manchester Museum for this specimen.

SUMMARY

Extracts of the livers of a number of species of animals and birds have been examined spectroscopically with particular reference to their contents of vitamins A_1 and A_2.

It has been found that, in general, mammal and bird livers contain vitamin A_1 but no vitamin A_2. In the 21 species examined the only exceptions found were the giant monitor (considerable vitamin A_1 and only traces of vitamin A_2) and animals known to feed on fish, i.e. the seal and the otter. A mammalian liver exceptional in that it contained more vitamin A_2 than vitamin A_1 was obtained by feeding a rat with a concentrate of freshwater fish liver. It would thus appear that although mammals cannot metabolize vitamin A_2 they can pass it into the liver if they receive it preformed into their digestive tract.

The vitamin A_1 contents of all the livers examined fell within the rather narrow limits of 0·7–32 mg. per 100 g. fresh liver (average for 18 species = 8·3 mg. per 100 g.) with the exception only of the python, giant monitor and otter with values of 86, 250 and 47 respectively.

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CXCVIII. VITAMIN B₁ AND COCARBOXYLASE IN ANIMAL TISSUES[1]

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THE discovery by Lohmann & Schuster [1937] that cocarboxylase is the pyrophosphoric ester of vitamin B₁ suggested that in animal tissues the vitamin is active like this ester in the oxidation of pyruvic acid. It now seems clear that animal tissues and certain bacteria do not decarboxylate pyruvic acid to acetaldehyde and CO_2 (as yeast does) but instead oxidize it to acetic acid and CO_2, either by dismutation under anaerobic conditions or directly [Krebs & Johnson, 1937; Lipmann, 1937, 1; Weil-Malherbe, 1937]. Lipmann [1937, 2] reports that with acetone preparations from *B. Delbrückii* the simultaneous decarboxylation and oxidation of pyruvic acid require cocarboxylase.

In animal tissues evidence that vitamin B₁ pyrophosphate is concerned in the oxidation of pyruvic acid is derived (1) from catatorulin tests, and (2) from the presence of cocarboxylase in tissues and their alleged capacity to synthesize cocarboxylase from vitamin B₁. The first point, that cocarboxylase can replace vitamin B₁ in catatorulin tests [Lohmann & Schuster, 1937], has not been confirmed in this laboratory using "teased" brain [Peters, 1937]; more recent unpublished experiments with brain slices also gave negative results. This point obviously requires further elucidation.

In regard to the second point, Auhagen [1932] first showed that boiled extracts of animal tissues stimulated the decarboxylation of pyruvic acid by yeast preparations (aetiozymase), indicating the presence of cocarboxylase. Simola [1932] investigated the influence of nutrition upon this phenomenon. Synthesis of cocarboxylase from vitamin B₁ by minced animal tissues or various tissue preparations has been reported from several laboratories [von Euler & Vestin, 1937; Tauber, 1937; Lohmann & Schuster, 1937; Peters, 1937], whereas Stern & Hofer [1937] reported negative results. This work is not yet sufficiently quantitative and further it has not so far been ascertained whether vitamin B₁ is present in tissues in the free form.

We have now developed a method which allows the separate quantitative estimation of cocarboxylase and free vitamin B₁, by means of which the following points have been investigated: (1) the cocarboxylase and vitamin B₁ contents of boiled extracts from normal and avitaminous tissues, (2) the enzymic synthesis of cocarboxylase from vitamin B₁. In the present paper we shall show that there is much more cocarboxylase than vitamin B₁ present in animal tissues, and that it is much reduced when vitamin B₁ is withheld from the diet, and further, that the liver readily synthesizes cocarboxylase from vitamin B₁ *in vivo*. Another paper will deal with the synthesis of cocarboxylase *in vitro*. Various organs (brain, muscle) have only a very limited power of synthesis;[2] intestinal mucosa does not show any activity at all, whereas active preparations can be obtained from the liver.

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[1] A preliminary report of this work appeared in the *Trans. Soc. Chem. Ind.* **57**, 470, 1938.

[2] Less in the case of brain than suggested by Peters [1937], who did not know of the stimulating effect of vitamin B₁ upon the action of cocarboxylase (cf. below).

EXPERIMENTAL

Dry baker's yeast (supplied by Distillers Co. Ltd.) has been used throughout. It was freed from cocarboxylase by rapid washing with $M/10$ Na_2HPO_4 (1 g. yeast 3 times with 50 ml. each time) and once with distilled water at room temperature.[1] The washed yeast was suspended in 10 ml. $M/10$ phosphate buffer of pH 6·2. The production of CO_2 from pyruvic acid was measured manometrically using both Barcroft and Warburg manometers. Each bottle contained 1·0 ml. washed yeast suspension (added last), 0·1 ml. $MgCl_2$ (0·1 mg. Mg), 0·4 ml. water or experimental solutions and 0·2 ml. sodium pyruvate adjusted to pH 6·2 (5 mg. pyruvic acid). The reaction was started after 10–12 min. by tipping in the pyruvate solution from the side bulb or dangling tube. The total volume of fluid was 1·7 ml., the temp. was 28° and the gas either air or nitrogen.

Preparations

Cocarboxylase. One pure specimen from Dr K. Lohmann (Berlin); another specimen (70 % purity, vitamin B_1-free) prepared by Mr H. W. Kinnersley from yeast crude cocarboxylase, obtained by enzymic synthesis from synthetic vitamin B_1 by the method of Kinnersley & Peters [1938].

Vitamin B_1 chloride hydrochloride. Synthetic specimens from Messrs Hoffmann la Roche and Messrs Bayer.

Pyrimidines and thiazole vitamin components. From Prof. A. R. Todd (Lister Institute, London).
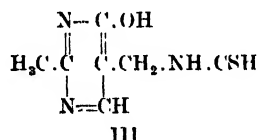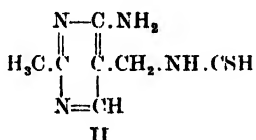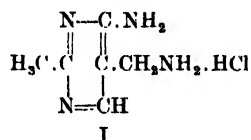
Vitamin B_1 analogue from 4-methyl-5-β-hydroxypropylthiazole (referred to in Table II as vit. B_1 analogue IV) from Dr Buchman (Pasadena).
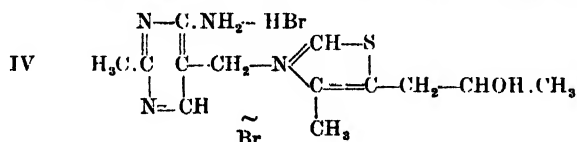
Cozymase. Prepared from yeast by the method of Meyerhof & Ohlmeyer [1937] by Mr L. A. Stocken in this laboratory; this was 91% pure by comparison with pure cozymase kindly supplied by Dr Ohlmeyer. Other compounds used came from commercial sources.

We are much indebted to those mentioned above for their kind gifts which have made this work possible.

1. The activation of cocarboxylase action by vitamin B_1

Whereas vitamin B_1 cannot replace cocarboxylase, it has been found by one of us [Ochoa, 1938] that it markedly stimulates the decarboxylation of pyruvic acid by alkaline washed yeast in the presence of pure cocarboxylase. The effect is apparent with 0·1 γ and increases with increasing concentrations of the vitamin;

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[1] The above method of washing was adopted when Lohmann's method failed. At first the latter gave good results.

with $1 \cdot 0\gamma$ cocarboxylase it is nearly maximal for 15γ vitamin B₁ hydrochloride. Fig. 1 illustrates this. The activation is the same in air as in N_2 showing that it is not due to oxidative removal of acetaldehyde. That the vitamin does not induce a disappearance of acetaldehyde by dismutation is shown by the fact
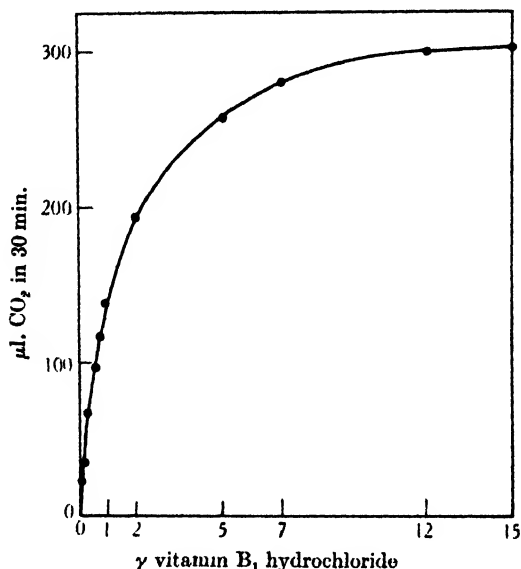
Fig. 1. Activation of cocarboxylase action by vitamin B₁. Ordinate μl. CO_2 evolved in presence of increasing amounts of vitamin and 1γ cocarboxylase, minus CO_2 evolved with 1γ cocarboxylase alone (1γ cocarboxylase $= 163\,\mu$l. CO_2; control $= 26\,\mu$l. CO_2 in 30 min.).

that no acid production takes place (measured in bicarbonate solution; gas, pure CO_2). Further, iodoacetic acid, while depressing the decarboxylation to some extent, has practically no effect on the activation by the vitamin, as shown in Table I.

Table I. *Action of iodoacetic acid on the stimulation of cocarboxylase by vitamin B₁*

		μl. CO_2 in excess of control in 30 min.			% inhibition of vitamin activation
Exp. no.	Molarity of IAA	1γ cocarb.	1γ cocarb. $+10\gamma$ vit. B₁	Activation by vit. B₁	
1	0	211	505	294	—
	0·0009	187	479	292	0
2	0	162	465	303	—
	0·0050	115	373	258	15

The stimulating effect of the vitamin is due to the pyrimidine part of the molecule and is absent in the absence of the NH_2 group. A number of other compounds investigated were found to be inactive (Table II).

Vitamin B₁ monophosphate has no cocarboxylase activity (as already stated by Lohmann & Schuster), whether alone or together with vitamin B₁, but it was found to stimulate cocarboxylase action. The monophosphate was obtained by acid hydrolysis of cocarboxylase (15 min. in N HCl at 100°); the solution was used for the experiments after bringing the reaction to pH 6·2 with NaOH (Table III).

Table II. *Effect of various compounds on the decarboxylation of pyruvic acid by alkaline washed yeast in the presence of pure cocarboxylase*

Exp. no.	Sample	μl. CO_2 evolved in 30 min.
1	Control	15
	1γ cocarboxylase	160
	1γ cocarb. $+2\gamma$ vitamin B_1	344
	1γ cocarb. $+20\gamma$ pyrimidine I	385
	1γ cocarb. $+50\gamma$ pyrimidine II	318
	1γ cocarb. $+90\gamma$ pyrimidine II	385
	1γ cocarb. $+90\gamma$ pyrimidine III	161
	1γ cocarb. $+20\gamma$ vitamin B_1 analogue IV	432
	1γ cocarb. $+90\gamma$ thiazole	150
	1γ cocarb. $+100\gamma$ cytidylic acid	168
	1γ cocarb. $+100\gamma$ vicine	158
	1γ cocarb. $+100\gamma$ adenosine	160
	1γ cocarb. $+100\gamma$ nicotinamide	153
2	1γ cocarboxylase	178
	1γ cocarb. $+50\gamma$ acetylcholine	174
	1γ cocarb. $+100\gamma$ choline	161
	1γ cocarb. $+100\gamma$ spermine	154

Table III. *Effect of vitamin B_1 monophosphate on the decarboxylation of pyruvic acid by alkaline washed yeast with and without addition of cocarboxylase. (Quantities expressed for vitamin B_1 monophosphate as γ cocarboxylase; 1γ cocarboxylase = 0.6γ vitamin B_1)*

Sample	μl. CO_2 evolved in 30 min.
Control	9
10γ vitamin B_1	14
10γ vitamin B_1 monophosphate	12
10γ vitamin $B_1 + 10\gamma$ vitamin B_1 monophosphate	14
1γ cocarboxylase	135
1γ cocarb. $+1\gamma$ vitamin B_1 monophosphate	188
1γ cocarb. $+2\gamma$,,	203
1γ cocarb. $+5\gamma$,,	257
1γ cocarb. $+10\gamma$,,	315

Recently Lipschitz *et al.* [1938], observing that addition of vitamin B_1 to alkaline washed yeast containing Mg^{++}, pyruvate and boiled tissue extract, increased the CO_2 output, interpreted this to mean that the vitamin is phosphorylated to cocarboxylase by the yeast enzymes in the presence of the extract. The results quoted above, however, indicate that the increased CO_2 production is due to the stimulation by the vitamin of the effect produced by the cocarboxylase present in the tissue extract. The fact that the pyrimidine components (which cannot be synthesized to cocarboxylase through lack of thiazole) also activate the cocarboxylase action makes it very unlikely that the effect of the vitamin itself is due to synthesis to cocarboxylase. That dry yeast does not phosphorylate vitamin B_1 to cocarboxylase to any extent is also indicated by the experiments of Kinnersley & Peters [1938]. The nature of the vitamin activation is not yet understood and is being further investigated.

Lipschitz *et al.* [1938] also reported that hexosediphosphate markedly increases the decarboxylation of pyruvic acid when boiled tissue extracts are present, an effect which is increased by addition of vitamin B_1; they supposed this action to be due to an influence on the enzymic synthesis of cocarboxylase from vitamin B_1. We find, however, that hexosediphosphate increases the CO_2 output in the complete absence of vitamin if both pure cocarboxylase and cozymase are present. The

effect increases with increasing concentrations of cocarboxylase[1] (Tables IV and V). Since accumulation of acetaldehyde is known to inhibit the enzymic decarboxylation of pyruvic acid the mechanism of action of hexosediphosphate is

Table IV. *Effect of hexosediphosphate on the decarboxylation of pyruvic acid by alkaline washed yeast in the presence of boiled extract from normal pigeon's liver, with and without addition of cocarboxylase. (Hexosediphosphate added, 7·8 mg. as Na salt)*

Sample	$\mu l.\ CO_2$ evolved in 2 hr.
Control	59
0·3 ml. boiled liver extract	235
0·3 ml. boiled liver extract + hexosediphosphate	293
0·3 ml. boiled liver extract + 0·5γ cocarboxylase	601
0·3 ml. boiled liver extract + 0·5γ cocarboxylase + HDP*	840

* HDP = Hexosediphosphate.

Table V. *Effect of hexosediphosphate and cozymase on the decarboxylation of pyruvic acid by alkaline washed yeast in the presence of pure cocarboxylase. (Hexosediphosphate added, 7·8 mg. as Na salt. Cozymase added, 0·2 mg.)*

	$\mu l.\ CO_2$ evolved in 2 hr.				
	γ cocarboxylase added				0·5γ cocarb. +5γ vit. B₁
Sample	0·5	1·0	2·0	3·0	
Control	48	34	36	73	46
Cocarboxylase	192	361	667	790	427
Cocarb. + HDP*	162	334	657	783	394
Cocarb. + Coz.†	182	354	657	1036	435
Cocarb. + HDP* + Coz.†	200	533	1085	1400	701

* HDP = Hexosediphosphate. † Coz. = Cozymase.
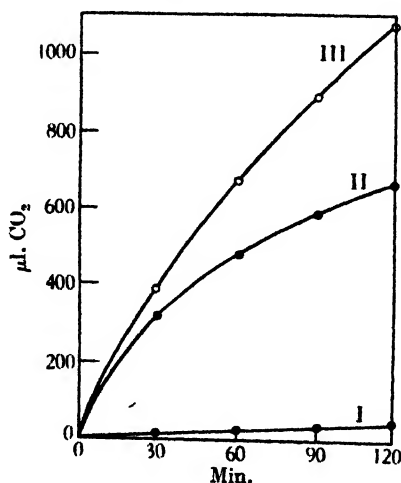
Fig. 2. Effect of hexosediphosphate and cozymase on the decarboxylation of pyruvic acid by alkaline washed yeast in presence of cocarboxylase. Curve I, control. Curve II, 2γ cocarboxylase. Curve III, 2γ cocarboxylase + 7·8 mg. Na hexosediphosphate + 0·2 mg. cozymase.

clear. The yeast enzymes form triosephosphate which dismutates with acetaldehyde in the presence of cozymase to give ethyl alcohol and phosphoglyceric

[1] Essentially the same results have been obtained with brewer's yeast (Löwenbräu, Munich).

acid, so that acetaldehyde is removed and the decarboxylation can proceed at a higher rate. That this effect of hexosediphosphate should be unaffected by fluoride and inhibited by iodoacetic acid [cf. Lipschitz *et al.* 1938] is obvious and requires no further comment. When tissue extracts are added they supply the necessary cocarboxylase and cozymase. Fig. 2 shows that, in presence of hexosediphosphate and cozymase, the production of CO_2 drops less rapidly than it does with cocarboxylase alone, so that the effect of hexosediphosphate markedly increases with time.

2. *Activation by manganese*

Lohmann & Schuster [1937] found that either Mg^{++} or Mn^{++} are necessary for the decarboxylation of pyruvic acid by yeast. Mn^{++} was found by the above authors to be 5–10 times as effective as Mg^{++}, the largest amounts investigated being 10 and 100γ respectively. We find now that whereas 100γ Mg^{++} produce practically a maximum effect, the activation brought about by Mn^{++} continues to increase above 10γ up to approximately 100γ and may be very large (Fig. 3).
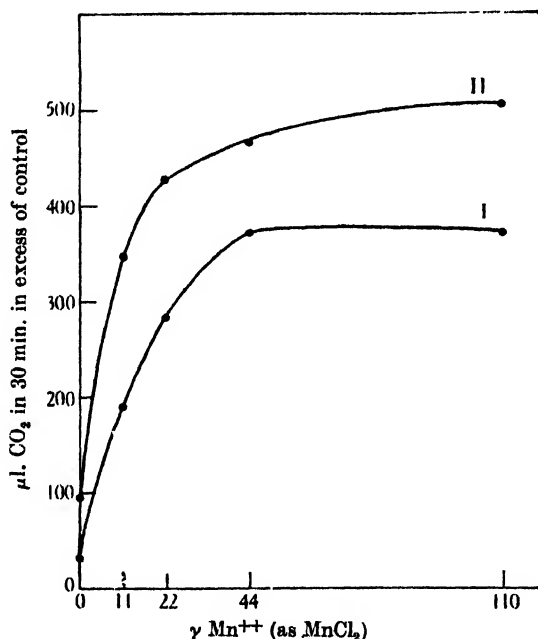
Fig. 3. Activation by Mn^{++} of the decarboxylation of pyruvic acid by alkaline washed yeast in presence of 1γ cocarboxylase, with and without vitamin B_1. Curve I, no vitamin. Curve II, 10γ vitamin B_1 hydrochloride.

This fact would only be of physiological significance if there is uneven distribution in the cell since 1 g. dry bottom yeast, Löwenbräu, Munich, contains only about 5.5γ Mn^{++} according to Ohlmeyer & Ochoa [1937], it is nevertheless interesting in regard to the mechanism of the reaction. Further, small amounts of Mn^{++} increase the activating effect of vitamin B_1. It should be noted that washing of our yeast by the method given above must remove most of the active ions, since if no Mg^{++} or Mn^{++} are added (in presence of cocarboxylase) no significant CO_2

production above the control (without cocarboxylase) takes place. This may account for the fact that if we add the Mg^{++} (100γ) mixed with the pyruvate, as Lohmann & Schuster did, we get very low CO_2 productions.

3. *Method of estimation of cocarboxylase and vitamin B_1*

The activation of the cocarboxylase action by vitamin B_1 makes it possible to determine the two compounds separately if present together. If an amount of vitamin B_1 which produces maximum (or nearly maximum) activation is added to the unknown solution, the cocarboxylase can be determined, since the CO_2 production will not be very much affected by the vitamin which was originally present. The addition of vitamin has also the effect of increasing very markedly the sensitivity of the method. Amounts of $0\cdot01$–$0\cdot02\gamma$ cocarboxylase can thus be estimated. For the cocarboxylase estimation $0\cdot1$ ml. vitamin B_1 solution in $M/10$ phosphate pH $6\cdot2$ (10γ vitamin B_1 hydrochloride) and $0\cdot3$ ml. unknown solution are added to the bottles, together with the other additions, and the CO_2 production is measured over a period of 30 min. The amount of cocarboxylase is found by reference to a curve obtained with pure cocarboxylase + 10γ vitamin B_1 (Fig. 4, curve II). In our experience, duplicates agree within the usual limits of
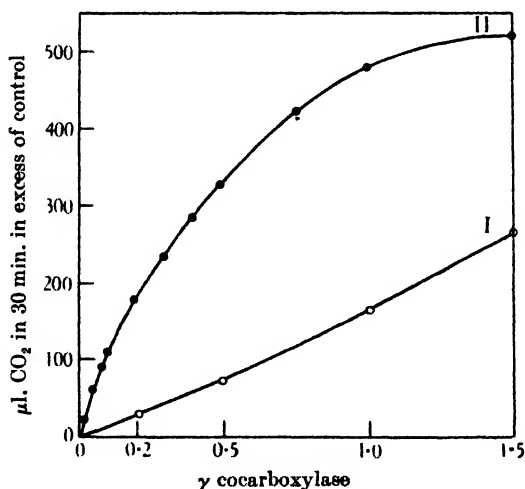
Fig. 4. Decarboxylation of pyruvic acid by alkaline washed yeast with increasing concentrations of cocarboxylase. Curve I, cocarboxylase alone. Curve II, cocarboxylase + 10γ vitamin B_1 hydrochloride.

error of manometric measurement. The assumption must be made that the action of boiled tissue extracts is due to the presence of cocarboxylase. This is at present reasonable as no other compound is yet known which has the same action as vitamin B_1 pyrophosphate.

For the estimation of vitamin B_1 the curve in Fig. 1 is used, working with a constant concentration of cocarboxylase. With an unknown extract, cocarboxylase is first determined, then extra cocarboxylase is added up to a total of $1\cdot0\gamma$ in the bottles used. In this way CO_2 in excess of that produced with $1\cdot0\gamma$ cocarboxylase alone represents CO_2 due to vitamin B_1 stimulation. The sensitivity of the vitamin estimation is much lower; amounts of vitamin under $0\cdot05\gamma$ cannot be detected under our present experimental conditions.

With Mg^{++} (100γ) it is often found that there is little difference between the action of 0.1 and 0.2γ vitamin. In presence of a trace of Mn^{++}, however, the difference becomes distinct as the following experiment shows.

$$\mu l. CO_2 \text{ in 30 min. } (3\gamma\ Mn^{++}\text{ as } MnCl_2.\ \text{No } Mg^{++})$$

0·5 cocarboxylase only	34·7 (triplicate)
0·5 cocarb. 0·1γ vitamin B₁	44·6 (triplicate)
0·5 cocarb. 0·2γ vitamin B₁	53·1 (duplicate)

This recent finding is being further explored.

Here the assumption that any stimulation by tissue extracts is due to vitamin B_1 cannot be made, as vitamin B_1 monophosphate would produce a similar effect. It is unlikely that free pyrimidines or other vitamin derivatives are present in sufficient concentrations to produce any effect. If, in what follows, we refer to free vitamin B_1 in tissue extracts, the possibility remains that the active substance may be partially or totally the monophosphate.

The main source of error in these methods is that the activity of the washed yeast may vary slightly from day to day or even from experiment to experiment, although washing and other experimental conditions have remained unaltered. It has been our custom therefore to check the cocarboxylase curve frequently and apply a correction if necessary. Some idea of the possible error may be got as follows: for a solution containing 0.25γ cocarboxylase and 1.3γ vitamin B_1 in 0.3 ml. an error of $\pm 0.02\gamma$ in estimating the cocarboxylase (an extreme case) might influence the vitamin estimation to the extent of $\pm 0.2\gamma$, the vitamin value thus becoming $1.3 \pm 0.2\gamma$. Our experience with yeast variations is curious. After initial rigid standardization, owing to the steady character of the results we relaxed our precautions of including known amounts of cocarboxylase and vitamin B_1 in each group of estimations; this saved much time. Latterly we have reinstated this precaution because at times unaccountable variations appear.

On the other hand, small variations in the amount of yeast in the bottles in a given experiment are without effect. A significantly lower CO_2 production (as compared with 1 ml.) has been found only with amounts of yeast suspension under 0·5 ml. as shown in Table VI.

Table VI. *Decarboxylation of pyruvic acid by alkaline washed yeast using various amounts of yeast suspension*

		$\mu l.\ CO_2$		
Sample	Time min.	1·0 ml. washed yeast	0·5 ml. washed yeast	0·25 ml. washed yeast
0·5γ cocarboxylase	30	72	73	56
0·5γ cocarb. + 10γ vitamin B₁	30	323	293	247
0·5γ cocarboxylase	60	111	115	91
0·5γ cocarb. + 10γ vitamin B₁	60	468	449	383

Recoveries of cocarboxylase and vitamin B_1 added together to various tissues are shown in Table VII. There is a tendency for rather high recoveries with small amounts of cocarboxylase in presence of large amounts of vitamin. Although this is not yet understood, the results show that no significant destruction of either compound takes place during the operations involved in the preparation of the boiled extracts. It must be realized that only 1/9[1] of the amounts added per g. tissue are actually estimated, i.e. in line 5 (Table VII) about 0.14γ cocarboxylase.

It may be added that the method suggested by Lipschitz *et al.* [1938] cannot lead to the desired separate determination of cocarboxylase and vitamin B_1, because it involves for the former the use of iodoacetic acid, which we have shown not to inhibit the vitamin activation, and for vitamin B_1 the use of

hexosediphosphate which can increase CO_2 production in the complete absence of free vitamin.

Table VII. *Recovery of cocarboxylase and vitamin B_1 added together to minced normal pigeon's tissues before preparing boiled extracts therefrom (cocarboxylase and vitamin B_1 are expressed in γ per g. fresh tissue)*

Tissue	Cocarboxylase, γ				Vitamin B_1, γ			
	Added	Found	Recovered	%	Added	Found	Recovered	%
Muscle	0·00	4·14	—	—	0·00	2·43	—	—
	0·75	5·20	1·06	141	18·70	19·50	17·07	91
	3·28	7·20	3·06	93	1·64	4·15	1·72	105
Liver	0·00	4·85	—	—	0·00	0·90	—	—
	1·30	7·02	2·17	167	32·50	32·40	31·50	97
	5·00	9·55	4·70	94	2·51	—	—	—
	0·00	4·38	—	—	0·00	0·99	—	—
	5·00	8·52	4·14	83	5·00	5·68	4·69	94
	2·50	7·30	2·92	117	25·00	20·70	19·71	79
	0·00	4·23	—	—	0·00	—	—	—
	4·00	8·18	3·95	99	8·00	—	—	—

4. *Cocarboxylase and vitamin B_1 contents of animal tissues*

In early experiments Simola [1932] found a decrease in cocarboxylase (about −60%) in the brain and liver of rats upon synthetic diets as compared with those on normal diets; in the absence of vitamin B the amounts were further reduced; the cozymase content did not vary. Westenbrink [1934] and Leong [1937] have reported estimates of vitamin B_1, by weight and bradycardia tests respectively, in rat tissues; presumably such determinations should include cocarboxylase. Leong's principal values were for rats upon ordinary diet: liver 3·75, heart 3·5, brain 3·5, muscle 1·0γ vitamin B_1 per g. tissue, upon the assumption that 1 international unit $= 2·5\gamma$ vitamin B_1.

Preparation of boiled extracts. Pieces of pigeon and rat tissues were weighed in weighing bottles, minced or chopped and ground in a mortar with 2 vol. distilled water; the suspension was then heated for 3–5 min. in a boiling water bath with stirring, cooled and centrifuged. The brain, as well as the liver, was sometimes minced on an ice-cold plate, but the results so obtained did not differ from those reached by mincing at room temperature. In the case of one animal the brain was removed under amytal anaesthesia after freezing *in situ* with liquid air [Kerr, 1935]; again there was no significant difference. We think therefore that cocarboxylase remains comparatively stable at room temperature, though we know that slight disappearance occurs on incubation at 38°.

Estimations have been performed upon 0·3 ml. samples of extracts from brain, muscle, liver and heart in animals taken as follows.

Pigeons.

I. Normal.

II. Vitamin B_1-deficient (with symptoms).

III. Rice-fed for 25 days (no symptoms).

IV. Vitamin B_1-deficient (with symptoms) and injected with vitamin B_1 (1 mg. per 100 g. animal) 25 min. before death.

V. Vitamin B_1-deficient (with symptoms) and dosed by mouth with vitamin B_1 (100γ *per diem* per animal) for 3 days before death.

VI. Rice-fed for 26 days (no symptoms) but treated as animals of group V for 3 days before death.

Rats.

    I. Normal.

    II. Vitamin B_1-deficient (with symptoms).

    III. Same as II but vitamin B_1 injected (1 mg. per 100 g. animal) 45 min. before death.

    IV. Normal or avitaminous but vitamin B_1 given by mouth (1 mg. per 100 g. animal) 1 to 2 hr. before death.

All animals were killed by decapitation and bled before taking tissue samples.

Birds which have been upon a polished rice diet for 25 days and over and which are receiving daily doses of vitamin B_1, are perfectly well in appearance but they do not regain weight significantly. This is due to lack of other nutritional factors [Carter & O'Brien, 1935; 1936]. They form a good control for the birds with symptoms because of their generally normal behaviour at a much diminished body weight (about 70 % of the normal). 100γ vitamin B_1 *per diem* is a comparatively large dose as 10γ would already produce curative effects.

Results. The average values for pigeons are given in Table VIII and individual values of rats in Tables IX and X. The values for pigeon's brain and liver are shown graphically in Figs. 5 and 6. Individual results for pigeons are given
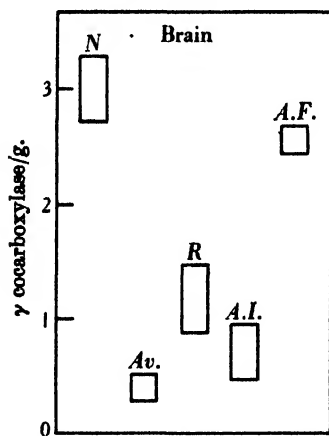
Fig. 5.   Fig. 6.
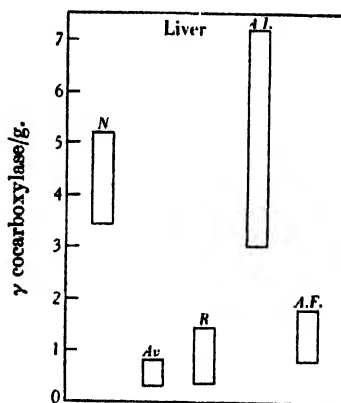
Fig. 5. Cocarboxylase in pigeon's brain. *N*, normal. *Av.*, avitaminous (with symptoms). *R*, rice-fed (no symptoms). *A.I.*, avitaminous after injection of vitamin B_1. *A.F.*, avitaminous fed vitamin B_1 for 3 days. Ordinates: mean $\pm 2\epsilon$.

Fig. 6. Cocarboxylase in pigeon's liver. *N*, normal. *Av.*, avitaminous (with symptoms). *R*, rice-fed (no symptoms). *A.I.*, avitaminous after injection of vitamin B_1. *A.F.*, avitaminous fed vitamin B_1 for 3 days.

in the appendix. $\pm 2\epsilon$ (the standard error of the mean) represents $\sigma/\sqrt{n}$ for 6 estimations and over, but $\sigma/\sqrt{(n-1)}$ for fewer estimations than 6. Values are calculated upon the assumption that the fresh tissues contain 70 % H_2O.

    A more accurate value would be for brain 80 %, liver 72 %, muscle 74 %, based upon the figures of Roche [1925]: brain, normal 80·7, avitaminous 79·9, inanition 80·2. Liver, normal 71·3, avit. 71·6, inan. 72·6. Muscle, normal 74·8, avit. 73·7, inan. 75·4. A few estimations by us confirm these values. The extreme variations for Roche's tissues were small. For truer values our brain results can be raised by 5 % approx. and the muscle values by 1·5 %.

Though we cannot claim close accuracy for our present method and think it possible that further work may bring to light other sources of error than those referred to above (p. 1503) we consider that our determinations are sufficiently good to demonstrate the points at issue. Even if 50 % of the cocarboxylase estimations (cf. Table VIII) were in error to the extent of $+50\%$ this would mean that the values in Table VIII would be higher by 25 %, but the conclusions below would still stand. As to the vitamin results we can obtain no evidence for the presence of free vitamin B_1 (or its monophosphate) in normal or avitaminous brain, whereas it can be detected after administration. This is therefore a clear difference, but in view of the lower sensitivity of the vitamin method (see p. 1507) it can only be stated definitely that there is less than 0.45γ per g. present.

In the case of the results with brain, and occasionally with liver, showing no vitamin B_1, it has sometimes happened that the tissue extract gave slightly less CO_2 than the control with 1γ cocarboxylase. This has been within the limits of experimental error, so that there is no reason to suspect the presence of inhibitory substances.

In the case of the cocarboxylase values for liver in group IV (Table VIII), it will be seen that they are upon the average over 10 times larger than the corresponding ones of group II. This cannot be due to error introduced by the presence of large amounts of free vitamin, since this (as judged from Table VII) could not raise the results more than 1·7 times.

The outstanding points to note in these results are:

(1) There is much less vitamin B_1 than cocarboxylase in the liver and brain.

(2) The cocarboxylase is reduced in all tissues in both the avitaminous and the rice-fed pigeon, as well as in the avitaminous rat.

Table VIII. *Cocarboxylase and vitamin B_1 in pigeon's tissues*
(*values expressed in γ per g. fresh tissue*)

Group	Condition of birds	Tissue	Cocarboxylase			Vitamin B₁		
			No. of obs.	Mean	Standard error ±2ε	No. of obs.	Mean	Standard error ±2ε
I	Normal	Brain	12	3·00	±0·28	7	0·00	±0
		Muscle	9	3·81	±0·42	7	1·60	±0·50
		Liver	9	4·33	±0·88	7	0·32	±0·30
		Heart	8	4·36	±0·57	5	1·54	±0·75
II	Avitaminous (symptoms)	Brain	9	0·40	±0·12	6	0·00	±0
		Muscle	6	1·04	±0·56	5	0·96	±0·71
		Liver	9	0·48	±0·30	7	0·20	±0·20
		Heart	6	0·55	±0·47	5	0·45	±0·27
III	Rice-fed (no symptoms)	Brain	8	1·18	±0·31	—	—	—
		Muscle	2	0·81	—	—	—	—
		Liver	3	0·73	—	—	—	—
		Heart	4	0·91	—	—	—	—
IV	Avitaminous, vitamin B₁ injected 25 min. before death	Brain	4	0·72	±0·24	4	0·55	±0·27
		Muscle	4	2·20	±0·37	4	3·04	±1·90
		Liver	4	5·10	±2·10	4	14·20	±10·20
		Heart	4	2·05	±0·52	4	4·05	±1·50
V	Avitaminous, dosed 3 days with vitamin B₁	Brain	6	2·55	±0·12	—	—	—
		Muscle	6	1·55	±0·20	—	—	—
		Liver	6	1·28	±0·51	—	—	—
		Heart	6	2·46	±0·49	—	—	—
VI	Rice-fed (no symptoms), dosed 3 days with vitamin B₁	Brain	4	2·82	—	—	—	—
		Heart	4	2·45	—	—	—	—

(3) There is a large increase in both cocarboxylase and vitamin B_1 in the livers of animals shortly after administration of vitamin.

(4) There is a much larger relative increase in the cocarboxylase in brain (and heart) than in the other tissues in pigeons dosed with smaller amounts of vitamin over a period of 3 days. In fact practically normal values are found.

(5) The brain cocarboxylase of pigeons not showing symptoms is higher than that of those which do show symptoms.

Table IX. *Cocarboxylase and vitamin B_1 in rat's tissues. (Individual values. Expressed in γ per g. fresh tissue)*

		Brain		Muscle		Liver		Heart	
Group and condition		Cocarb.	Vit. B_1	Cocarb.	Vit. B_1	Cocarb.	Vit. B_1	Cocarb.	Vit. B_1
I	Normal	2·93	0·00	0·38	0·90	5·40	0·60	4·15	—
	,,	2·91	0·20	0·32	0·50	4·85	0·60	3·50	0·60
II	Avitaminous	0·68	—	0·10	—	0·53	---	0·52	---
	,,	0·53	—	0·11	—	0·36	—	0·31	—
III	Avitaminous,	0·72	—	0·16	3·80	6·30	24·30	0·74	—
	vitamin B_1 in-	0·54	0·50	0·13	—	2·91	84·60	0·67	—
	jected	0·73	—	0·20	1·10	8·62	7·40	0·77	---

Table X. *Cocarboxylase and vitamin B_1 in the liver of rats after oral administration of vitamin B_1 (Group IV); individual values*

Condition	Animal killed after (hr.)	Cocarboxylase γ per g.	Vitamin B_1 γ per g.
Normal	1	6·50	3·40
Avitaminous	2	6·30	8·10
Avitaminous	2	8·40	8·30

DISCUSSION

We interpret the above facts to mean that cocarboxylase is a very significant form of vitamin B_1 in animal tissues. In some, such as brain and liver, it appears to be predominant; in skeletal and heart muscle there is relatively more vitamin B_1 present, which sometimes approaches the amount of cocarboxylase, hence we cannot say dogmatically that vitamin B_1 itself may not also have some normal function in life. In rat muscle the amount of cocarboxylase is surprisingly low, but there is not much vitamin B_1 either; this agrees with the findings of Westenbrink [1934] and Leong [1937]. As will be noticed by comparing groups II, IV and V of Table VIII, vitamin B_1, if administered, is taken up by the liver and rapidly synthesized to cocarboxylase; with small doses over a period of several days the amount present in the liver again decreases and a preferential increase takes place in the brain and heart, i.e. in the two tissues which are specially affected by vitamin B_1 deficiency. There must therefore be a special affinity for heart and brain tissues reflected in their biological needs. It is evident that even considerable reduction (down to 40 % of the normal) in the cocarboxylase content of the brain can occur before the function of its cells is impaired as shown by the appearance of symptoms. So far as this implies that these symptoms are associated with a low brain cocarboxylase, the evidence reinforces previous views from this laboratory as to the dependence of normal function upon biochemical state. But it is a new point that the liver may co-operate.

What we now want to know is whether in normal functioning there is any local synthesis by brain tissue or whether all the cocarboxylase is transported as such in the blood. The fact that in some birds there is a massive synthesis of

cocarboxylase in the liver before the symptoms clear up is not decisive, because either any local synthesis might be slow, or the cells might take an appreciable time to re-establish sufficient normality.

The finding of Sinclair [1938] that the vitamin B_1 in the blood is in a combined form, though admitting of other interpretations, is consistent with its conveyance as the pyrophosphoric ester; nevertheless there is difficulty in understanding how such an ester could permeate brain and other tissue cells, a difficulty not lessened by the failure to confirm the equal catatorulin activity of cocarboxylase.

We may ask whether the intestine phosphorylates vitamin B_1 *in vivo* in a similar fashion to that suggested for glucose and fat by Verzár & McDougall [1936] or for lactoflavin by the experiments of Rudy [1936]. This needs some direct test, but the experiments of Table X indicate that much vitamin B_1 as such can reach the liver; they are therefore indirect evidence against obligate phosphorylation in the intestine. This conclusion is supported by the failure to obtain *in vitro* phosphorylation of the vitamin with intestinal mucosa, as already referred to in the introduction (unpublished experiments). There seems to be little doubt that the proof that the liver participates in the metabolism of this vitamin is important and suggestive from the clinical standpoint. Its full understanding must wait for more accurate knowledge of the metabolism of pyruvic acid.

SUMMARY

1. Vitamin B_1 (as also does its monophosphoric ester) stimulates the decarboxylation of pyruvic acid by alkaline washed yeast in the presence of cocarboxylase. This action is due to the pyrimidine half of the molecule containing the NH_2 group. It is not due to removal of acetaldehyde by oxidation or dismutation. The nature of this activation is not yet understood and is being further investigated.

2. Hexosediphosphate increases the rate of decarboxylation in presence of cozymase and pure cocarboxylase in the total absence of vitamin B_1. The mechanism of this effect is discussed.

3. Mn^{++} greatly stimulates the carboxylase system if present in sufficient concentrations.

4. The activation of cocarboxylase action by vitamin B_1 has been made use of in a method for the separate estimation of cocarboxylase and free vitamin in the same solution or tissue extract.

5. There is much less vitamin B_1 than cocarboxylase present in boiled extracts from rat and pigeon brain and liver: both occur in those from muscle and heart tissues.

6. The cocarboxylase content of tissues is much reduced in the B_1-avitaminous condition and specifically so in the brain tissue, being soon increased in the latter (and also in the heart) after a short period of administration of the vitamin.

7. Administration of vitamin B_1 to animals leads to an immediate accumulation of both vitamin B_1 and its pyrophosphoric ester in the liver. For the first time this brings the liver into prominence in the metabolism of vitamin B_1.

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APPENDIX

Table XI. *Cocarboxylase and vitamin B₁ in tissues from normal pigeons (γ per g.)*

Brain		Muscle		Liver		Heart	
Cocarb.	Vit. B₁	Cocarb.	Vit. B₁	Cocarb.	Vit. B₁	Cocarb.	Vit. B₁
3·60	—	4·90	—	2·70	—	4·60	—
3·40	0	3·40	0·90	4·05	0·50	5·22	0·80
2·44	0	—	—	—	—	—	—
2·61	0	—	—	—	—	—	—
2·60	0	2·97	2·25	2·80	0·54	2·90	2·70
2·60	—	4·50	1·60	5·00	—	5·20	1·30
2·96	—	3·70	1·60	3·95	0·00	4·05	—
2·96	—	3·33	0·80	4·40	0·30	5·10	—
3·87	—	2·87	1·80	7·20	0·00	3·38	1·80
3·42	0	4·95	—	4·05	0·00	4·46	1·08
2·76	0	—	—	—	—	—	—
2·70	0	—	—	—	—	—	—
—	—	4·14	2·43	4·85	0·90	—	—

Table XII. *Cocarboxylase and vitamin B₁ in tissues from avitaminous pigeons (γ per g.)*

Brain		Muscle		Liver		Heart	
Cocarb.	Vit. B₁	Cocarb.	Vit. B₁	Cocarb.	Vit. B₁	Cocarb.	Vit. B₁
0·54	—	2·35	—	1·00	—	1·70	—
0·45	0	0·90	1·20	0·31	0·60	0·36	0·54
0·35	0	1·08	2·10	0·18	0·50	0·45	0·72
0·18	—	0·63	0·40	0·22	0·00	0·18	0·00
0·27	0	0·65	0·50	0·15	0·00	0·21	0·54
0·18	0	0·63	0·60	·0·34	0·00	0·40	0·45
0·72	0	—	—	0·23	0·30	—	—
0·40	0	—	—	1·50	0·00	—	—
—	—	—	—	0·40	—	—	—
0·54	—	—	—	—	—	—	—

Table XIII. *Cocarboxylase in tissues from rice-fed pigeons
(no symptoms) (γ per g.)*

Brain	Muscle	Liver	Heart
0·99	—	0·45	—
2·20	0·54	0·31	0·99
0·93	1·08	1·44	0·63
0·99	—	—	0·68
0·90	—	—	1·35
1·22	—	—	—
0·95	—	—	—
1·28	—	—	—

Table XIV. *Cocarboxylase and vitamin B₁ in tissues from avitaminous pigeons
a short time after injection of vitamin B₁ (γ per g.)*

Brain		Muscle		Liver		Heart	
Cocarb.	Vit. B₁	Cocarb.	Vit. B₁	Cocarb.	Vit. B₁	Cocarb.	Vit. B
0·84	0·40	2·50	5·40	5·75	19·00	2·50	3·60
0·72	0·40	2·07	2·70	2·34	9·50	2·07	4·50
0·42*	0·50	2·40	2·70	5·93	24·00	1·44	5·60
0·90*	0·90	1·80	1·35	6·30	4·50	2·20	2·50
0·81†	0·00	1·71	0·90	2·34	2·70	2·34	2·70
0·54‡	—	0·90	—	1·35	—	0·54	—

* Not recovered at the end of 25 min.
† Bird killed after 1 hr. Not included in average.
‡ Only 25γ vitamin B₁ HCl injected. Killed 25 min. after recovery. Not included in average.

Table XV. *Cocarboxylase in tissues from rice-fed pigeons (19–27 days, with
symptoms) dosed by mouth for 3 days with 100γ vitamin B₁ per day*

Date	No. days on rice	Initial wt. g.	Final wt. g.	Wt. after B₁ dosing	γ cocarboxylase per g.			
					Brain	Muscle	Liver	Heart
28 April	27	340	202	203	2·70	1·86	0·81	1·90
	27	402	262	258	2·75	1·26	2·11	1·80
	27	473	316	328	2·43	1·35	0·86	2·70
20 May	19	340	231	232	2·54	1·41	0·92	3·20
	19	371	244	255	2·43	1·35	0·90	3·02
	27	280	194	212	2·43	2·03	2·07	2·12
Average		368	241	248				
Av. % fall		—	65	67				
Av. % increase		—	—	2				

Table XVI. *Rice-fed birds dosed with 100γ vitamin B₁ on
successive days*

No. days on rice	Initial wt. g.	Final wt. g.	Wt. after 3 days dosing	γ cocarboxylase per g.	
				Brain	Heart
26	357	252	274	3·40	2·30
26	388	270	299	2·75	1·90
26	395	278	293	2·25	2·90
26	316	210	221	2·90	2·70
Average	364	252	272	2·82	2·45

Av. % fall 69. Av. % rise 6.

Note added 23 August 1938. Similar results to ours for cocarboxylase in
tissues have been reached simultaneously by Westenbrink & Goudsmit (*Nature*,
1938, **142**, 151).

CXCIX. IMPROVEMENTS IN THE USE OF THE FORMALDEHYDE AZO REACTION FOR VITAMIN B_1

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THIS paper embodies further observations made upon and in relation to the formaldehyde azo reaction for vitamin B_1 [Kinnersley & Peters, 1934] and its possible application to semi-quantitative estimation of vitamin B_1. The development of the more sensitive thiochrome method of estimation [Jansen, 1936; Karrer & Kubli, 1937; Pyke, 1938] during the progress of this work, may ultimately make the method here described obsolete. Nevertheless, we think that some independent check may prove useful, and further, that the method may prove of value, when enough vitamin B_1 is available, owing to the stability of the standards, and to the fact that a dark room and a source of ultra-violet light are not required. Unfortunately, neither method is successful with phosphoric esters of vitamin B_1; these do not give the formaldehyde azo test (as stated by Lohmann & Schuster [1937]); they form a blue fluorescent compound with alkaline ferricyanide, but this does not pass into a butyl alcohol layer, which is a basis of separation from interfering fluorescent substances [Kinnersley & Peters, 1937]. We have found that phosphatase can be used in the pre-treatment of the extracts to liberate the vitamin B_1. In our previous communication we stated that the condition of alkalinity chosen and the presence of formaldehyde largely reduced interference by other substances; a particularly troublesome impurity giving a pseudo-vitamin B_1 colour reaction could be distinguished by its relative instability in acid solution. We have realized for some time that butyl alcohol would extract the bulk of the pink substance formed in the reaction. By an error the colour was compared in our previous paper [1934] with phenol red in phosphate buffer pH 5; it should have been "approximately pH 7·3". A considerable improvement is again made by shaking the butyl alcohol with dilute acid, when the pink substance passes back into the acid aqueous phase; the whole test now compares favourably as regards specificity with other methods and can be readily applied to vitamin B_1 concentrates from the International Standard Clay (1936) and also the cruder "charcoal" concentrates. We have found certain conditions which interfere with this and other azo tests. The work falls into two parts:

I. The method and some conditions modifying it;
II. Application to crude vitamin B_1 extracts.

I. *The method and some conditions modifying it*

(a) *The original formaldehyde azo test has been modified as follows.*
To 0·1–0·3 ml. of a solution containing 10–20γ vitamin B_1 (acidity best about pH 6·0) and 30 % ethyl alcohol, is added after one minute a mixture of 1·25 ml. of the special reagent and 0·5 ml. diazotized sulphanilic acid. The alcohol now included has proved essential for full development of the pink colour [cf. Koessler & Hanke, 1919]. After standing for 2 hr. or more, the pink-coloured

solution is extracted twice successively with 2 ml. of butyl alcohol. The combined butyl alcohol extracts are then extracted with 2 ml. $N/200$ HCl and with 1 ml. twice (this should make the pH of the aqueous phase about 6·5). If not acid after shaking, more acid (trace of $N/10$) must be added: the pink HCl phase is now removed, and an equal volume of ethyl alcohol is added. The resulting coloured solution is compared with standards of vitamin B$_1$ similarly treated. They should be kept out of strong light except during tests. We have tried the colorimeter (visual and photoelectric), but are convinced that equal and even better accuracy in these dilute solutions is obtained by a naked eye comparison of similar small bore tubes. Our estimate of the accuracy of the method is $\pm 5\%$ for amounts of aneurin HCl of approximately 20γ. Standards of 40γ and over can be prepared.

(b) *Conditions modifying the test*

During our experience with this test some facts have been discovered which are briefly recorded. Reducing agents such as cysteine or traces of sodium hydrosulphite, markedly delay the appearance of colour. Traces of some metals interfere both with azo reactions of the Pauly type for histamine and histidine, and also with the reaction for vitamin B$_1$.[1] Other systems have not been investigated. We do not know of any previous publication showing this effect upon azo reactions.

Histidine. Estimating 20 μg. by the usual Pauly reaction according to the technique of Koessler & Hanke [1919], no interference was produced by U, Mn, Nb, Rb, Ta, Be, Pb, La, Th, Thallium, V, Cs, Cr, Fe in amounts of 20γ, or by Al 10γ, or In 25γ: interference was produced by Ni 5γ, Co 10γ, Hg 20γ, Mn slightly, Cd 100γ, Sn 9γ, Ce 20γ slight, Zn 10γ, Cu 3γ. The metals interfere mostly by reducing pinkness in the solution.

Vitamin B$_1$ formaldehyde azo reaction. Remarkably little interference was produced by most metals tried. Exceptions were found in Cu, Hg, Ag. With 10γ vitamin B$_1$ Cl, HCl, 4γ Hg were inhibitory and 10γ Ag slightly so. The effect of Cu was of the greatest interest. A delayed reaction developed with minute traces of this, an amount equivalent to one atomic weight per mol. producing practically maximal effect. The action depends upon the presence of formaldehyde.

The question arises whether the presence of a metal could explain the delayed azo reaction previously reported by Kinnersley *et al.* [1935] during the formation of quinochromes by oxidation with manganese oxides. If Mn compounds could produce the effect this would be reasonable; but they do not do so, nor is it possible to modify the reaction for vitamin B$_1$ by addition of the solution in which the vitamin is oxidising. Hence the original interpretation that the delayed azo reaction indicates a change in the vitamin molecule is still tenable.

II. *Application of reaction to crude vitamin B$_1$ extracts*

We have made a preliminary attempt to apply this to certain food materials, as well as to vitamin B concentrates, using for biological tests the birds required in the course of another research. After considerable trial, 50% acid ethyl alcohol has been employed to extract the crude, ground foodstuff. Both vitamin B$_1$ and its pyrophosphoric ester are very soluble in 50% ethyl alcohol and stable in faintly acid solution; 50% acid alcohol also removes vitamin B$_1$ from adsorbents such as charcoal. From what is now known of the chemistry of these substances it seems to us to be inconceivable that this extraction is not adequate; tests with 75% alcohol have not shown any larger recovery of the vitamin. In our experience it is essential to concentrate the vitamin and to remove it from certain interfering substances. For this purpose we cannot improve upon sodium phosphotungstate, which has been long used in this laboratory for the preparation of

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[1] Some of these reactions were shown to the Biochemical Society in May 1936.

vitamin B_1 and which is known to precipitate it in very low concentration. The complete method is as follows.

A convenient amount (100 g. in the case of oatmeal) of the finely ground foodstuff is treated with 1000 ml. of boiling water; 2 ml. conc. HCl are added to bring it to pH 3·5, and the whole is heated for a few minutes to boiling. The rather thick mixture is placed on the boiling water bath for an hour or more and enough ethyl alcohol is then added gradually to make it up to 50 % by volume. The whole is filtered after cooling. Some materials require more solvents than others. The extract, after cooling overnight, and being freed from precipitate by Büchner filtration, is evaporated to 150 ml.; the water bath suffices for this as this vitamin is relatively stable in dilute acid solution. After further cooling and removal of insoluble matter by the centrifuge, Na phosphotungstate, 10 % at pH 6, is added to slight excess and the mixture is acidified to pH 1·0 with 20 % H_2SO_4. The phosphotungstate precipitate is allowed to stand 12 hr., collected in the centrifuge tube, and ground with baryta three times, according to our usual technique [Kinnersley et al. 1935]. After removal of baryta by H_2SO_4, the extract is concentrated if necessary at pH 3, care being taken to see that greater acidity does not develop during concentration; any free H_2SO_4 should be removed by $BaCl_2$ during this process. A convenient volume not exceeding 0·3 ml. is taken for the test.

Table I. *Comparison of vitamin B_1 in food materials by colour and biological tests.* (γ *per g. or ml. test material undried; no. of birds used in parentheses*)

		γ vitamin estimated by		Bird test		
Food material	Moisture content %	Colour test	Bird test	g. food given	γ vitamin found	$\pm 2\epsilon\ (\gamma)$
1. Yeast concentrate 41	—	120·0	145·00 (153)			
2. Wheat germ	7·0	20·0	13·50 (11)			
3. Pea	11·65	3·5	2·94 (81)	6	1·95 (11)	
				3	3·27 (45)	2·72–3·97
				5	2·80 (25)	2·22–3·55
4. Breakfast oats	10·0	3·0	2·54 (48)	6	2·23 (16)	
				5	2·75 (12)	
				3	2·67 (20)	2·39–2·98
5. Haricot bean	12·7	2·2	2·15 (5)			
6. Oatmeal	10·3	2·0	1·87 (41)	6	2·00 (20)	
				5	1·75 (21)	
7. Maize	9·7	2·0	1·54 (55)	6	1·41 (26)	1·09–1·82
				10	1·12 (6)	
				5	1·80 (23)	
8. Lentil	12·7	1·75	1·60 (12)			
9. Barley	12·0	1·2	0·71 (11)			

NOTES:

1. Yeast concentrate 41 was a 50 % alcohol concentrate from charcoal used in routine work in the Laboratory.

2. The average day dose for the birds is calculated by the logarithmic method [Kinnersley & Peters, 1936].

3. The day dose is taken as equivalent to 2·5γ.

4. The individual tests are not here included, in order to economize space. They can be made available by writing to this laboratory.

5. In a few cases, the values of $\pm 2\epsilon$ are quoted as a guide. For barley there was wide variation in the 11 tests of which the average is given.

6. Values for assays of vitamin B_1 quoted in the review by Fixsen & Roscoe [1938] are wheat germ 10–55γ, pea 1·0–3·0γ, haricot bean 1·3–2·5γ, oatmeal 8γ, breakfast oats 3·5γ, whole maize 0·75–1·5γ, lentil 1·0–5·2γ, all per g.

Using the pigeons available in the laboratory in the course of another research, we have obtained the following preliminary results. So far as we know the curative and protective test is valid for these cruder concentrates, though Kinnersley & Peters [1936] showed that it would not apply to the pure vitamin. The figures given in columns 5 and 6 of Table I give reasonably satisfactory agreement between the larger and smaller doses except in the case of the 6 g. dose for the pea, but here it will be noted that the number of tests is fewer. Inspection of Table I shows that parallel results are obtained with the azo method and biological tests; the order of arrangement in the table is based on the colour test and it agrees with that obtained by the bird test. The results of the bird tests have been calculated upon the assumption that one day dose equals $2 \cdot 5 \gamma$ vitamin B_1; in 1936, we concluded that one day dose must be equivalent to $2 \cdot 0 \gamma$ *per diem* as the result of tests of the catatorulin type and also of colour reactions. Since then, however, we have found that our extract of the acid clay may have deteriorated and that a better value is $2 \cdot 5 \gamma$ (cf. Sampson & Keresztesy [1937], also Pyke [1938]). Though there are some marked deviations from previous estimates of similar foodstuffs in the literature (see note 6 to Table 1), our results as a whole are of the same order.

Phosphoric esters of vitamin B_1 (cocarboxylase etc.). We have attempted to decide how far the presence of phosphoric esters of vitamin B_1 interfere in the case of (a) yeast extracts and (b) foodstuffs. The most suitable method for breaking up the phospho-compounds is enzymic. After considerable trial, and with the final advice of Dr Kay to whom we are grateful, we adopted takaphosphatase (as present in takadiastase $0 \cdot 5 \%$), acting at approximately pH 4. The result of one experiment is given in Table II.

Table II. *Effect of takadiastase on cocarboxylase (crude)*

Exp. 1. Solutions A, B, C were made up as stated below and incubated at 38° for one hour, after which the vitamin B_1 and phospho-esters were determined by a thiochrome method upon an aliquot. The results in the Table are expressed as equivalents of vitamin B_1.

Solution	A	B	C
Takadiastase	100 mg. in 10 ml.	Nil	100 mg. in 5 ml.
Cocarboxylase	20 ml.	10 ml.	Nil
Phosphate buffer pH 4	50 ml.	25 ml.	12·5 ml.
Water	Nil	5 ml.	Nil
Content in esterified or free vitamin B_1			
Before incubation:			
Vitamin B_1: Esterified	600	600	Nil
Free	100	100	Nil
After incubation:			
Vitamin B_1: Esterified	50*	500	Nil
Free	500*	100	Nil
% "free" vitamin liberated by phosphatase	91	15	—

* These values were checked by azo colour tests which were in agreement. The crude cocarboxylase solution contained preformed vitamin B_1.

Both by azo test and fluorescence it was found that approximately 90% of the combined vitamin B_1 had been converted into the free form by this treatment.

Application of this treatment to yeast extracts showed that in the initial extracts combined as well as free vitamin B_1 might be present, but there was no reason to think that this applied to the foods. Hence this possible objection to the use of both the azo test and the modification of Jansen's thiochrome methods in these foodstuffs appears in practice to be without foundation.

Though vitamin B_1 added can be recovered quantitatively, it is conceivable that some of the vitamin B_1 present in various forms in the foodstuffs may escape extraction with dilute acid or precipitation by phosphotungstate. But any such amounts are likely to be small in view of the known properties of the vitamin and its compounds. In any case, a method of this nature is valuable chiefly to establish a minimum. In view of the fair parallelism seen in Table I, we think that the method merits further exploration.

SUMMARY

1. The formaldehyde azo test for vitamin B_1 has been improved and extended to include a preliminary investigation of the vitamin content of foodstuffs, in which colour tests have been compared with biological tests. Parallel results have been obtained.

2. Certain metals in small amounts interfere with this test, and also with reactions of the Pauly type.

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CC. THE IDENTITY AND MECHANISM OF ACTION OF THE GLYCOTROPIC (ANTI-INSULIN) SUBSTANCE OF THE ANTERIOR PITUITARY GLAND

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THE experiments of Cope & Marks [1934] were the first to demonstrate that the injection of a crude anterior pituitary extract into a normal rabbit can induce complete insensitivity to the hypoglycaemic action of insulin administered many hours after the last injection of pituitary extract. In the experiments of these authors the blood sugar level of the treated rabbit was seldom significantly affected by the injections of the anterior pituitary preparation alone, and the "anti-insulin" activity of the extracts was therefore not due to a "diabetogenic" action. For the purpose of convenience the name "glycotropic factor" was suggested [Young, 1936, 1] for the anterior pituitary substance which induces insulin insensitivity without itself causing a rise of blood sugar level. Investigation showed [Young, 1936, 1] that the glycotropic factor was present in preparations of prolactin obtained by the method of Riddle *et al.* [1933], a fact which appeared to be of significance with respect to the action of anterior pituitary preparations on milk secretion [Young, 1936, 1, 2]. Subsequent observations [Young, 1937, 1; 1938] showed that prolactin and the glycotropic substance are not identical. The evidence presented in support of this differentiation, although conclusive, has previously been described only briefly, and it is one purpose of the present communication to supplement the description of the earlier work and to present further evidence that prolactin and the glycotropic factor are not identical. The results of experiments on the influence of the administration of anterior lobe extracts on the hyperglycaemic action of injected adrenaline and on sugar tolerance are also recorded in the present paper, and the significance of the results obtained is discussed with respect to the mechanism of action of the glycotropic substance.

EXPERIMENTAL

Animals

The animals used in the majority of the experiments in the present investigation have been unanaesthetized, healthy, young adult rabbits, male or female, weighing about 1·5 kg., from an inbred pure strain of Dutch rabbits bred at the farm laboratories of the National Institute for Medical Research. In our experience animals of this strain are particularly constant in their response to glycotropic pituitary extracts.

In the investigations on the influence of the subcutaneous injection or intravenous infusion of adrenaline it was convenient to use larger rabbits, so pure strain Chinchilla rabbits, and occasionally rabbits weighing about 3 kg. purchased from a dealer, were used for these experiments.

[1] Work begun during the tenure of a Beit Memorial Fellowship.

All the rabbits received an ample diet of cabbage, bran, oats and hay. For experiments on the glycotropic factor it is essential to use rabbits which are in excellent condition and are eating well, in order to obtain consistent results.

Biological assays

Thyrotropic activity was assayed by the method of Rowlands & Parkes [1934]. The thyrotropic activity considered in the present investigations is therefore that causing an increase in size of the thyroid gland of the immature guinea-pig. It is not certain that the substance active in this respect is identical with that stimulating secretory activity of the thyroid gland.

Prolactin was assayed by the pigeon crop-gland method according to the technique described by Rowlands [1937]. The name prolactin is used to describe the substance active in causing increase in weight of the pigeon crop-gland, without reference to the question whether or not all preparations of this substance are truly lactogenic [cf. Folley & Young, 1938].

Method of expressing the results of biological assays

The question of establishing International standard preparations of prolactin and of the thyrotropic hormone, and of establishing International units of activity in terms of these preparations, is at present under discussion. Any method of expressing the physiological activity of pituitary substances in terms of units, in the present paper, must therefore be regarded as of temporary value only. In connexion with the question of establishing an International standard preparation of prolactin, Dr O. Riddle and Dr R. W. Bates have kindly made available a preparation of prolactin standardized by them. An assay curve for this prolactin, which contains 10 Riddle units/mg., has been constructed by the methods used in this laboratory. This curve has allowed us to express the results of our pigeon crop-gland assays in terms of Riddle units, and in the present paper this has been done.

Rowlands & Parkes [1934] defined a unit of thyrotropic activity as that amount of thyrotropic substance which, when injected daily for 5 days, would induce the thyroid glands of a 200 g. guinea-pig to double in weight. This unit has been adopted in the present paper, and will be described as the Rowlands-Parkes unit of thyrotropic activity.

Blood sugar estimations

Blood sugar was determined on 0·1 ml. of blood by the method of Hagedorn & Jensen. The figures in the present paper are therefore higher than those for "true" blood sugar.

In our experience the blood sugar level of a pituitary-treated rabbit is very easily raised on handling unless great care is exercised not to excite the animal. It is necessary to ensure that the rabbit is perfectly comfortable during the taking of blood samples. The ear vein from which samples are to be drawn is pierced some hours before blood is required for sugar estimations, so that frequent small samples can be obtained without the slightest reaction on the part of the rabbit.

In most instances the blood sugar curves given in the present paper represent the average response for 6 or more rabbits. Average, rather than individual, results are given because small individual variations are thereby minimized. The groups of animals for which average curves are presented sometimes constituted only a small proportion of the total number of rabbits used in a particular type

of experiment, and the individual experiments for which average results are given were chosen at random from the total number available. Where the difference between the average curves for two different groups might or might not be significant, standard errors of corresponding values in the average curves were calculated. In this way it was possible to determine whether or not corresponding points in the two average curves differed significantly.

Adrenaline infusion

Adrenaline was infused into the marginal ear vein of the unanaesthetized rabbit following the technique of Cori *et al.* [1930], and using a Burn-Dale burette pump. 1/1000 adrenaline (Parke Davis Ltd.) was diluted to a suitable volume with 0·9 % NaCl solution containing 1 mg. of glutathione/5 ml., the latter being added to stabilize the dilute solution of adrenaline.

Anterior pituitary extracts

Extracts of ox, pig and horse pituitary glands were used in the present investigation. Most of the extracts were obtained from ox pituitary gland, however, and where the species-source is not stated it is to be assumed that the preparation was extracted from ox anterior lobe.

All the ox pituitary preparations were extracted from anterior lobe tissue which had been dissected as free as possible from posterior lobe tissue. In a number of instances, however, extracts of pig and horse pituitary tissues were made from the undissected glands.

(*a*) *Acetone desiccation of anterior lobe tissue.* Acetone desiccation of frozen ox anterior lobes was effected by mincing the frozen tissue into 20 vol. of ice-cold acetone. After remaining in acetone in the cold room overnight, the tissue was transferred at intervals of 24 hr. to 3 or 4 fresh portions of cold acetone. The dehydrated tissue was finally washed with ether and preserved in a vacuum desiccator at room temperature. Frozen pig or horse pituitary tissue was desiccated in acetone at room temperature. Commercial (B.D.H. Ltd.), acetone-desiccated ox anterior lobe tissue was used for a number of preparations.

(*b*) *Preparation of prolactin.* Six different types of prolactin were used. These differed in the source (ox, horse or sheep pituitaries; fresh gland or acetone-desiccated powder) and in the methods of extraction. The different types are distinguished by suffixes.

Prolactin (1) was obtained from fresh, frozen dissected glands by the method described by Young [1938]. The material designated Prolactin-C in the earlier paper is the Prolactin (1) of the present communication. In brief, the method of preparation consisted of alkaline extraction of the anterior lobe tissue at 0°, followed by precipitation and reprecipitation of the prolactin at pH 5·5. All these procedures were carried out in the cold room at 0° as far as possible, and the prolactin was stored in a frozen condition at $-11°$, in a solution of such concentration that 3 ml. were equivalent to 1 g. of fresh anterior lobe tissue.

Prolactin (2) was prepared from commercial acetone-desiccated ox anterior lobe by the method of Bates & Riddle [1935] using either 60 % or 70 % alcohol for the initial extraction, though a slight addition to the Bates-Riddle technique was made. This consisted in a number of reprecipitations from water at pH 5·5 of the material insoluble at pH 3·5 in the presence of Na_2SO_4. In this way any Na_2SO_4 present in the original precipitate was removed. The product was then dried and passed through the final stage of the Bates-Riddle method of purification.

Prolactin (3) was prepared from acetone-desiccated fresh gland by a method essentially that of Riddle *et al.* [1933], i.e. aqueous extraction at pH 8 followed by precipitation (and reprecipitation a large number of times) of the prolactin fraction at pH 5·5. The prolactin was finally washed with alcohol and ether and dried.

Prolactin (4) was similar to Prolactin (3) except that it was extracted from commercial dried gland.

Prolactin (5) was prepared by the slight modification of the Bates-Riddle method described by Young [1938]. The modification consisted in the replacement of the final fractionation with 70 % alcohol in the presence of NaCl by 6–12 reprecipitations from a large volume of water at pH 5·5. The product was washed with alcohol and ether and dried.

Prolactin (6) was the product obtained by an application to Prolactin (5) of a method of purification based on that of Lyons [1937]. 1 g. Prolactin (5) was stirred for 1 hr. at room temperature with 40 ml. $N/20$ HCl contained in 80 % aqueous acetone; the residue was extracted twice in the same manner. The combined extracts were then brought to the point of maximum precipitation by the addition of strong ammonia solution (about 0·25 ml.) and two volumes of alcohol were added. After settling overnight the precipitate was filtered off and dissolved in water at pH 8, any material insoluble at this pH being spun off and discarded, and it was then reprecipitated at pH 5·5. This process of solution at pH 8 and precipitation at pH 5·5 was repeated, and the precipitated prolactin was washed with alcohol and ether and dried.

(c) *Preparation of thyrotropic extracts.* The material which remained in solution when the prolactin fraction from ox pituitary tissue had been precipitated "isoelectrically" during the preparation of Prolactins (1), (2), (3) and (4) constituted the thyrotropic fractions used in the present investigation. No attempt was made to separate the thyrotropic and gonadotropic hormones, and all the thyrotropic preparations used possessed gonadotropic activity.

In each instance a thyrotropic preparation is numbered in agreement with the corresponding prolactin preparation.

Thyrotropic (1), i.e. the pH 5·5-soluble material corresponding with Prolactin (1), is the fraction described by Young [1938] as "non-prolactin-C". This was not precipitated and dried, but stored in solution by freezing at $-11°$. The solution was of such strength that 3 ml. of it were equivalent to 1 g. of fresh gland tissue.

Thyrotropic (2), the thyrotropic fraction corresponding with Prolactin (2), was prepared and dried according to the method previously described [Young, 1938]. This fraction was designated "ordinary non-prolactin" in the previous paper.

Thyrotropic (3) and *Thyrotropic* (4) were precipitated by the addition of 6 vol. of cold alcohol, washed with alcohol and ether and dried *in vacuo*.

(d) *Average yields of the different fractions.* The yields of the different types of preparation used are given in Table I as percentages of acetone-desiccated anterior pituitary tissue.

(e) *Posterior lobe contamination of anterior pituitary preparations.* As the administration of posterior pituitary extracts is known to affect carbohydrate metabolism, it was necessary to be certain that the anterior pituitary preparations used were not grossly contaminated with posterior lobe principles. The results of assays for oxytocic activity (guinea-pig uterus method) on the different preparations used are given in Table II. In a few instances the pressor activity of the ox pituitary preparations was determined, and found to be close to the figure expected from the previously determined oxytocic activity.

Table I. *Yields of the different pituitary preparations used*

Source of gland	Method of preparation	Yield, expressed as % of desiccated anterior lobe	
		Prolactin fraction	Thyrotropic fraction
Ox	(1)	— *	---*
	(2)	1·0– 1·6	0·8–1·3
	(3)	9·8–11·1	2·2–2·7
	(4)	5·3– 9·3	2·2–3·0
	(5)	6·7–12·8	—
	(6)	About 2	—
Horse	(3)	8·0–12·0†	—
Pig	(3)	11·7–14·2†	—

* These fractions were not dried before use but stored frozen in solution.
† Yield expressed as % of whole pituitary gland.

Table II. *Oxytocic activity of pituitary preparations*

(The preparations were heated in a boiling water bath with 0·25% acetic acid for 2 min. before assay.)

Source of gland	Method of preparation	Oxytocic activity (international units)	
		Prolactin fraction	Thyrotropic fraction
Ox	(1)	About 0·005 units/ml.	About 0·006 units/ml.
	(2)	<0·0001 units/mg.	0·25–0·75 units/mg.
	(3)	<0·0001 ,,	<0·0001 ,,
	(5)	<0·0001 ,,	—
	(6)	<0·0001 ,,	—
Horse	(3)	About 0·0003 ,,	—·
Pig	(3)	About 0·0001 ,,	—

The results in Table II show that with the exception of Thyrotropic (2) the ox pituitary preparations used possessed negligible oxytocic activity.

RESULTS

1. *The influence of pituitary extracts on the hypoglycaemic action of insulin*

In many early experiments of the present investigation on the glycotropic action of anterior pituitary extracts, 2 injections of a suitable extract were given daily to a rabbit for 2–4 days, and the response to 2 units of crystalline insulin, injected intravenously after a fast of 21 hr., was determined on the day following the last injection of extract. In these experiments the blood sugar curve following the administration of insulin was usually of the form illustrated in Fig. 1 [cf. Young, 1936, 1]. Occasionally, however, the daily injection of the anterior pituitary extract itself raised the blood sugar level of the rabbit [cf. Young, 1938] so that on the 3rd or 4th day of injection the blood sugar was at a definitely diabetic level. In these instances a glycaemic response to the injection of insulin, somewhat resembling that illustrated in Fig. 1, was sometimes, but not always, obtained (Fig. 2). In later experiments only two injections of anterior pituitary extract were given, these being administered at the beginning and at the 16th hour of a short fast. Two units of crystalline insulin were injected intravenously at the beginning of the 21st hour of fasting, and the blood sugar level was determined at intervals. With suitable pituitary extracts the hypoglycaemic action of the insulin was completely abolished (Fig. 3), there being no sign of an

initial, transient hypoglycaemia. Indeed, in most instances the immediate effect of the insulin injection was a definite rise of blood sugar level to 3–5 mg./100 ml. above its original level. Such a rise was not present when a volume of saline equal to that of the insulin solution was injected intravenously into the pituitary-treated rabbits. It should be mentioned that in spite of the fact that crystalline insulin (Burroughs Welcome) was used, a slight initial hyperglycaemic response to the intravenous injection of insulin was frequently observed in the control experiments on rabbits which had not been previously treated with pituitary extracts.
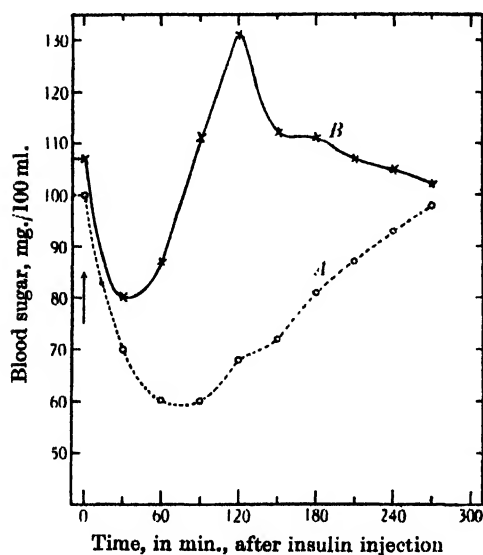
Fig. 1.
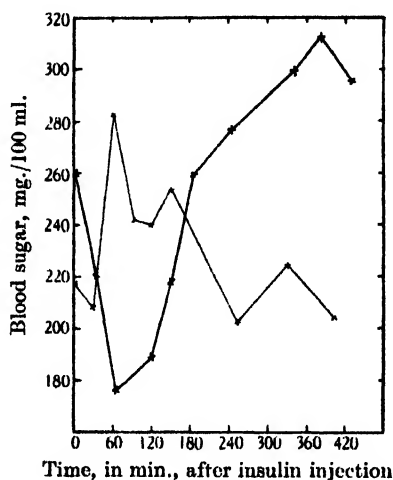
Fig. 2.

Fig. 1. The influence of treatment with the glycotropic substance on the hypoglycaemic action of insulin in the rabbit. *Curve A*. Average blood sugar response to the intravenous injection of 2 units of crystalline insulin of 7 normal rabbits which had fasted 18 hr. *Curve B*. Average blood sugar response to the same dose of insulin of the same rabbits after each had received 4 injections of glycotropic substance during 2 days.

Fig. 2. The response to the intravenous injection of 2 units of crystalline insulin of two rabbits which exhibited hyperglycaemia as the result of treatment with the glycotropic substance.

It should be pointed out that the experiments which yielded curves of the type shown in Fig. 1 were those in which the injections of the glycotropic substance were given over a period of some days, during which time the rabbits were feeding normally. Curves of the type shown in Fig. 3 were obtained in experiments with rabbits which received pituitary injections during a short period of fasting, but it is not clear how far the condition of the animal (fasting or fed) at the time the injections of pituitary extract were given, determined the type of response to insulin.

In all the above experiments insulin was administered intravenously, but it should be mentioned that similar results were obtained when insulin was injected subcutaneously [cf. Marks & Young, 1938].

Control experiments with extracts of muscle, liver, adrenal and pineal tissues, prepared in a manner similar to that for the extraction of pituitary gland, showed no detectable glycotropic activity in these tissues, under the conditions of our experiments (Fig. 4—Curve A).

Rabbits treated with glycotropic pituitary extracts have more liver glycogen at the end of a short fast than do control animals [Young, 1937, 2; Marks & Young, 1938]. Control experiments were therefore made on rabbits in which the fasting liver glycogen level was raised. It was found that if our rabbits were given

Time, in min., after insulin injection

Fig. 3.

Time, in min., after insulin injection

Fig. 4.
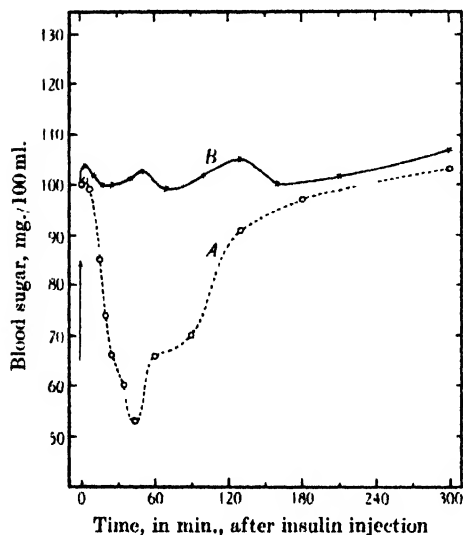
Fig. 3. The influence of treatment with the glycotropic substance on the hypoglycaemic action of insulin in the rabbit. *Curve A.* Average blood sugar response to the intravenous injection of 2 units of crystalline insulin of 6 normal rabbits which had fasted 21 hr. *Curve B.* A typical response to the intravenous injection of 2 units of crystalline insulin of a rabbit which had received 2 injections of glycotropic substance during the preliminary fast of 21 hr.

(In order to facilitate comparison, the blood sugar results are expressed as a percentage of the initial level. In the control group the initial level was 103 mg./100 ml. while in the treated animal it was 106 mg./100 ml.)
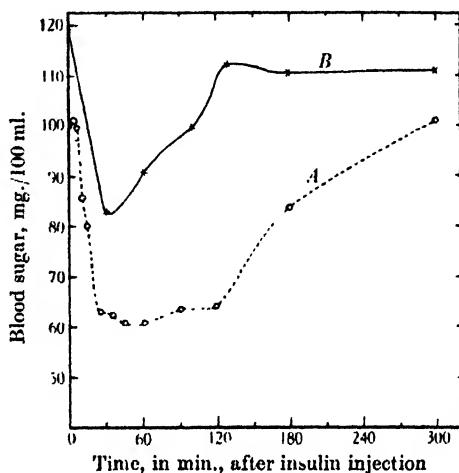
Fig. 4. The influence of treatment with non-specific tissue extracts and of feeding with glucose, on the hypoglycaemic action of insulin in the rabbit. *Curve A.* Average blood sugar response to the intravenous injection of 2 units of crystalline insulin of 6 rabbits which had received 2 injections of various tissue extracts during the preliminary fast of 21 hr. *Curve B.* Average blood sugar response to the intravenous injection of 2 units of crystalline insulin, of 18 rabbits which had received 24 g. of glucose by mouth at the beginning of the preliminary fast of 21 hr.

24 g. of glucose in 40 ml. of water by stomach tube at the beginning of a 21 hr. fast, the liver glycogen at the end of the fast averaged 3·2 % as compared with 1·8 % for untreated rabbits, and 5·1 % for rabbits treated with glycotropic substance (Prolactin (1)) during the period of fasting. The blood sugar level of the glucose-fed rabbits was slightly, but significantly, raised, as the average initial blood sugar in 18 experiments was 118·0 ± 2·4 mg./100 ml.,[1] compared with a normal value (50 animals) of 103·6 ± 2·3. However, a comparison of Curve A,

[1] 2·4 is the standard error of the mean value 118·0. The standard error of the mean is calculated from the formula $\epsilon = \sqrt{\dfrac{\epsilon(\bar{x}-x)^2}{n(n-1)}}$ where $\bar{x}=$ the mean value of the series of observations, $x=$ any individual observation, and $n=$ the number of observations. The difference between two mean values is taken to be significant if the value of $\dfrac{\bar{x}_1-\bar{x}_2}{\sqrt{\epsilon_1^2+\epsilon_2^2}}$ is greater than 3.

Fig. 3, with Curve B, Fig. 4, shows that in these animals the hypoglycaemic action of insulin was but little altered.

When the glycotropic activity of pituitary extracts was tested according to the later technique described above, the blood sugar level of the rabbits was never more than very slightly raised by the injections of the pituitary extracts alone, provided that the animals had been handled very carefully, and the extracts were of a suitable type. In 50 such experiments with active preparations, the average blood sugar level immediately before the insulin injection was 107.4 ± 2.4 mg./100 ml. while in 50 untreated control animals, which had likewise fasted for 21 hr., the blood sugar level averaged 103.6 ± 2.3. These results show that the administration of the anterior lobe extracts alone had no significant effect on the fasting blood sugar level of rabbits under the conditions of these experiments.

In order to facilitate the comparison of the glycotropic activities of different pituitary preparations, it was desirable to adopt a "unit" of glycotropic activity. As rabbits appear to be somewhat less sensitive to the anti-insulin action of glycotropic extracts during the middle of the summer, the results of assays carried out during the winter months only were used in this connexion. A unit of glycotropic activity was defined as that amount of extract which, on subcutaneous administration to a fasting rabbit in two equal doses according to the method described above, would just abolish, or almost abolish, the hypoglycaemic action of 2 units of crystalline insulin administered intravenously after a fast of 21 hr. The hypoglycaemic action of the insulin was considered to be almost abolished if the blood sugar level failed to fall more than 10 mg./100 ml. below its original value within 3 hr. of the insulin injection. An approximate value for the minimum amount of extract required could usually be determined in 3–5 experiments. Although no great quantitative merit can be claimed for the results of these assays, the differences observed between the activities of different types of extract were of such a magnitude as to be demonstrable by this method.

The evidence that prolactin and the glycotropic substance are different springs from two main sources: (a) a consideration of the widely differing glycotropic activities of different prolactin preparations obtained by different methods from pituitary glands of the same species, and by the same method from pituitary glands of different species; (b) the preparation of glycotropic extracts possessing no detectable crop gland-stimulating activity.

(a) *The relative glycotropic activities of different preparations of prolactin.* Table III gives the average prolactin and glycotropic activities of different prolactin preparations. It will be seen that Prolactin (6), although highly active as a pigeon crop gland stimulator, possessed no detectable glycotropic activity. On the other hand, Prolactin (1), extracted from pituitary glands of the same

Table III. *Average prolactin and glycotropic activities of prolactin preparations obtained by different methods*

Source of hypophysis	Method of extraction	Prolactin content Riddle units	Glycotropic activity units	Prolactin units / Glycotropic units
Ox	(1)	15/ml.	8/100 ml.	190
	(2)	6/mg.	12/g.	500
	(3)	3·5/mg.	6/g.	580
	(4)	0·5/mg.	1/g.	500
	(5)	3·5/mg.	5/g.	700
	(6)	6/mg.	<1/g.	>6000
Horse	(3)	0·2/mg.	12/g.	17
Pig	(3)	0·3/mg.	6/g.	50

species, possessed substantial glycotropic activity. Horse and pig pituitary tissues are poor in prolactin activity, but possess glycotropic activity comparable with that of ox pituitary tissue. It will be seen from Table III that the maximum value of the ratio prolactin activity/glycotropic activity (6000) is over 350 times the minimum value (17), which shows that there can be no relationship between the pigeon crop gland activity and glycotropic activity, i.e. prolactin and the glycotropic factor cannot be identical.

From the data in Tables II and III one can calculate the total prolactin and glycotropic activities of 100 g. of acetone-desiccated ox pituitary gland, as indicated by the results for the different preparations. The results obtained, which are given in Table IV, enable a comparison to be made of the different proportions of the total activities present in the desiccated gland which are carried through into these preparations. Prolactin preparations (1), (3) and (5) contain similar proportions of the total prolactin activity present in the gland tissue, but Prolactin (1) contains at least twice the total glycotropic activity of the other preparations. Prolactin (1) is obtained by a method involving only one extraction of the anterior lobe tissue [Young, 1938], so that a loss of active material must occur in the primary extraction. This probably accounts for the lower proportion of the total crop gland-stimulating material found in Prolactin (1), but renders the greater proportion of glycotropic activity found in this fraction more striking.

Table IV. *Prolactin and glycotropic activities contained in 100 g. of acetone-desiccated ox anterior lobe, as calculated from the data for the different specimens of prolactin*

	100 g. of ox acetone-desiccated gland calculated to contain	
Method of extraction	Prolactin Riddle units	Glycotropic activity units
(1)	22,500*	120*
(2)	7,800	16
(3)	36,400	63
(4)	3,650	7
(5)	34,000	49
(6)	12,000	<2

* Calculated on the assumption that 5 g. of fresh tissue = 1 g. of acetone-desiccated gland.

(b) *The preparation of glycotropic extracts possessing no detectable crop gland-stimulating activity.* A number of preparations of the thyrotropic hormone from ox pituitary gland were found to possess substantial glycotropic activity. The relevant data are summarized in Table V.

Table V. *Glycotropic activity of preparations of the thyrotropic hormone*

(All preparations extracted from ox hypophysis.)

Method of preparation	Thyrotropic activity Rowlands-Parkes units	Glycotropic activity units	Prolactin activity Riddle units
(1)	2/ml.	5/100 ml.	<0·3/ml.
(2)	1·5/mg.	<1/g.*	<0·03/mg.
(3)	1·0/mg.	5/g.	—
(4)	0·3/mg.	<1/g.*	—

* These preparations were contaminated with posterior lobe hormones. The results of the glycotropic tests are therefore unreliable (see below).

As preparations of the thyrotropic hormone which contain no amount of prolactin detectable by our methods possess substantial glycotropic activity, it is

again emphasized that prolactin and the glycotropic substance cannot be identical.

(c) *Differentiation of the glycotropic substance from the thyrotropic and gonadotropic hormones.* A number of the prolactin preparations which possessed glycotropic activity contained only traces of the thyrotropic and gonadotropic hormones. In particular Prolactin (2) and Prolactin (5) possessed so little thyrotropic activity that the administration of 20 mg. daily for 5 days failed to induce any enlargement of the thyroid gland of the immature guinea-pig under the conditions of our test. Similarly, the intravenous administration of 100 mg. of preparations of these types to an oestrous rabbit failed to cause ovulation. The amounts of thyrotropic and gonadotropic hormones contaminating these prolactin preparations must therefore have been very small, although their glycotropic activities were substantial. It is clear that there is no relationship between the gonadotropic and thyrotropic activities of pituitary extracts and their glycotropic activity, so that the glycotropic substance cannot be identical with the gonadotropic and thyrotropic hormones.

(d) *Differentiation of the glycotropic substance from the oxytocic and pressor substances of the posterior pituitary gland.* As seen above, anterior lobe extracts which contain only a trace of the oxytocic substance possess substantial glycotropic activity. Fig. 5 gives the average blood sugar response to 2 units of
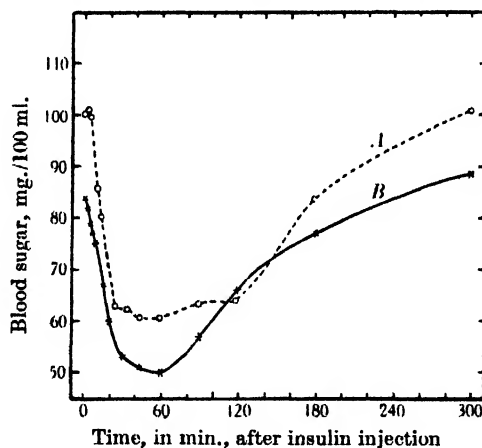
Fig. 5. The influence of treatment with pituitrin on the hypoglycaemic action of insulin in the rabbit. *Curve B.* Average blood sugar response to the intravenous injection of 2 units of crystalline insulin, of 6 rabbits each of which had received 2 injections of 30 units of pituitrin (Parke Davis) during the preliminary fast of 21 hr.

Curve A is the control curve from Fig. 4, which is inserted for the purpose of comparison.

crystalline insulin in a group of 6 rabbits, each of which received 2 injections of pituitrin (pressor + oxytocic substance—Parke Davis) containing together 60 units of oxytocic activity (Curve B). The average response to the same dose of insulin in control experiments (Curve A, Fig. 4) is included for comparison. The initial blood sugar level of the animals treated with posterior lobe extract was usually slightly below that of control animals (average value in 6 experiments = 84.0 ± 5.3), but the hypoglycaemic action of insulin was normal, the blood sugar level falling to about 60 % of its initial value in both groups of rabbits.

Burn [1923] found that when a posterior lobe extract and insulin were simultaneously injected subcutaneously into a rabbit, the hypoglycaemic action

of the insulin was almost abolished. Many other investigators have also found that pituitrin administration inhibits the hypoglycaemic action of subcutaneously administered insulin and depresses the sugar tolerance [cf. Cohen & Libman, 1937]. As Lambie & Redhead [1929] point out, the circulatory changes produced by the injection of pituitrin are profound and these may greatly modify the rate of diffusion into the tissues of injected material. The depressed rate of absorption of subcutaneously administered insulin may possibly account partly for the inhibitory action of simultaneously administered pituitrin. In the present investigation the second dose of pituitrin was given some hours before the intravenously injected insulin, and it is clear that the results of these experiments are not directly comparable with those of Burn and others. Our results do show, however, that the anti-insulin activity of the anterior lobe extracts was not due to the presence of traces of the oxytocic and vasopressor substances of the posterior lobe.

Anterior lobe extracts heavily contaminated with the oxytocic and vasopressor substances have always failed to exhibit glycotropic activity, even when there was reason to suspect that glycotropic substance was present in the extract. In a number of instances it was also found that the glycotropic activity of anterior lobe preparations was diminished when posterior lobe extracts were added to them. All these results are explicable on the assumption that when the anterior lobe extracts are heavily contaminated with the oxytocic and vasopressor substances, the diminished rate of absorption of the anterior lobe extracts caused by the intense vasoconstriction decreases the effectiveness of the administered glycotropic substance.

2. The influence of injections of the glycotropic factor on the sugar tolerance curve in the rabbit

It was briefly reported last year [Young, 1937, 1] that in a rabbit which has received 2 injections of a suitable preparation of the glycotropic substance and which has thereby become insensitive to the hypoglycaemic action of 2 units of insulin, the sugar tolerance curve is not significantly affected. Fig. 6 gives the

Time, in min., after glucose injection
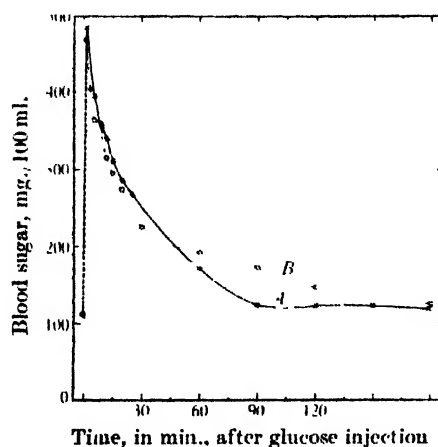
Fig. 6. The influence of treatment with the glycotropic substance on the sugar tolerance curve in the rabbit. *Curve A.* The average blood sugar response to the intravenous injection of 2 g. of glucose in 10 ml. of water of 6 normal rabbits which had fasted 21 hr. *Curve B.* The average blood sugar response to the same intravenous dose of glucose of the same rabbits after each had received two injections of the glycotropic substance during the preliminary fast.

average blood sugar response of a group of 6 rabbits which had fasted 21 hr. to the intravenous injection of 2 g. of glucose in 10 ml. of water, administered at 0 min. (Curve A). Curve B is the average response of the same group after each rabbit had received 2 injections of a glycotropic pituitary extract (in most instances Prolactin (1)) which contained in all 1 unit of glycotropic activity. The blood sugar response to the intravenous administration of 2 g. of glucose is remarkably little affected by previous treatment with an amount of glycotropic substance which induces complete insensitivity to the hypoglycaemic action of insulin. For the first hour after the injection of glucose there is practically no difference between the average fall of blood sugar level in the control group of animals and that in the group treated with the glycotropic extract. The blood sugar in the pituitary-treated rabbits does not reach quite such a high level as it does in the control animals, and appears to fall a little more quickly for the first 60 min., though no difference between the two curves is statistically demonstrable. After 60 min. the blood sugar level in the control group falls faster than that of the experimental group so that at 90 min. a difference exists between the two curves which is possibly statistically significant $\left(\frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\epsilon_1{}^2 + \epsilon_2{}^2}} = 2\cdot6 \right)$. The subnormal rate of fall of blood sugar in the experimental group, beginning about one hour after the glucose injection, could be explained in two ways. First, the glycogen stores of a rabbit which has received injections of a glycotropic extract during a 21 hr. fast are considerably higher than those of control animals [Young, 1937, 1]. It is therefore possible that one hour after the injection of glucose the glycogen stores of the pituitary-treated animals are so full that a diminution in the rate of glycogen storage occurs with a consequent decreased rate of removal of sugar from the blood. Second, as Fisher et al. [1936] have shown, pituitary extracts may exert a depressing action on carbohydrate oxidation during a fast. If, in the normal animal, the fall of blood sugar for about one hour after glucose is predominantly due to storage of glycogen, while the subsequent fall is primarily associated with carbohydrate oxidation, then the observed facts would find an explanation, though it must be confessed that at present there seems little evidence to support such a theory.

If a crude saline extract of ox anterior lobe is used as the source of glycotropic activity, the sugar tolerance of the injected rabbit may be slightly, though definitely, decreased. This is also true for a number of cases in which Thyrotropic (1) is used, but does not detract from the value of the observation that the administration of other preparations of glycotropic factor, in a dose which is sufficient to abolish the hypoglycaemic action of 2 units of insulin, has very little effect on the sugar tolerance curve. It is probably of significance that those extracts (crude saline extract and Thyrotropic (2)) which are sometimes found to diminish the sugar tolerance in the rabbit under the conditions of the above test, are those which were found to be diabetogenic in the dog [Young, 1938].

Himsworth & Scott [1938, 1] have recently investigated the influence of various conditions on the increase in sugar tolerance which occurs in the rabbit receiving a high carbohydrate diet as the result of consecutive intravenous injections of 1 g. of glucose at intervals of half-an-hour (Staub-Traugott effect). They found that the hypophysectomized rabbit receiving a high carbohydrate diet showed a Staub-Traugott effect, in that each successive injection of glucose resulted in a smaller rise of blood sugar level than the previous one. When the hypophysectomized rabbit received preliminary treatment with a preparation of the glycotropic substance, the sugar tolerance was diminished, as shown by the absence of Staub-Traugott effect when successive injections of glucose were

given. The impairment of sugar tolerance was similar to that which occurs when a normal rabbit receives a diet low in carbohydrate. In view of the results of Himsworth & Scott with hypophysectomized rabbits it was necessary to determine whether or not the Staub-Traugott effect was abolished in an intact rabbit treated with the glycotropic substance. Although extensive investigations have not been carried out on this point, the results show that, as with hypophysectomized rabbits, the Staub-Traugott effect is abolished in normal rabbits by treatment with the glycotropic substance (Fig. 7). This decrease of tolerance,
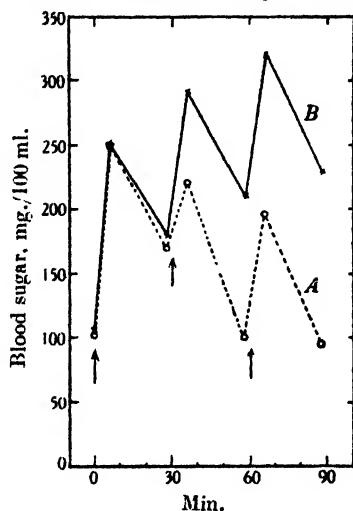
Fig. 7. The influence of treatment with the glycotropic substance on the sugar tolerance in the rabbit. *Curve A*. The blood sugar response to three intravenous injections of 1 g. of glucose, administered at half-hourly intervals to a normal rabbit. *Curve B*. The blood sugar response in the same rabbit after two injections of the glycotropic substance. 1 g. of glucose in 10 ml. of water was injected at the times indicated by the arrows.

which is explicable on the basis of either of the suggestions put forward above, is similar to that which occurs in rabbits fed on a low carbohydrate diet [Himsworth & Scott, 1938, 1]. The sugar tolerance of rabbits which have been rendered completely insensitive to the hypoglycaemic action of injection of insulin by treatment with the glycotropic substance, is therefore only very slightly decreased, and remains within the range of variation to be expected in normal rabbits receiving different diets.

3. *The influence of injections of the glycotropic factor on the hyperglycaemic response to adrenaline in the rabbit*

Corkill et al. [1933] and Cope & Marks [1934] found that in the rabbit hypophysectomy greatly decreased the hyperglycaemic action of subcutaneously administered adrenaline, and conversely, that the administration of a crude anterior lobe extract greatly increased the blood sugar response to the subcutaneous injection of adrenaline. Administration of a suitable preparation of prolactin to the normal rabbit also greatly increases the glycaemic response to the subcutaneous injection of adrenaline [Young, 1936, 1, 2].

Curve A (Fig. 8) gives the average hyperglycaemic effect of the subcutaneous injection of 20 μg. of adrenaline in a group of 10 normal rabbits which had fasted for 21 hr. Curve B (Fig. 8) is the average response of the same group to the same

dose of subcutaneous adrenaline after each rabbit had received two injections of prolactin (totalling 1 unit of glycotropic activity/rabbit) during the preliminary period of fasting. The difference in average response in the two groups is statistically significant, e.g. at 120 min. the average blood sugar level of the control group was 121 ± 3.5 mg./100 ml. while that for the experimental group was 165 ± 14; similar results were obtained for other points in the middle of the curves. An increase in the hyperglycaemic response to the subcutaneous injection of adrenaline was also found in a rabbit which had received injections of the glycotropic substance twice daily for 2–4 days.

Fig. 8.

Fig. 9.
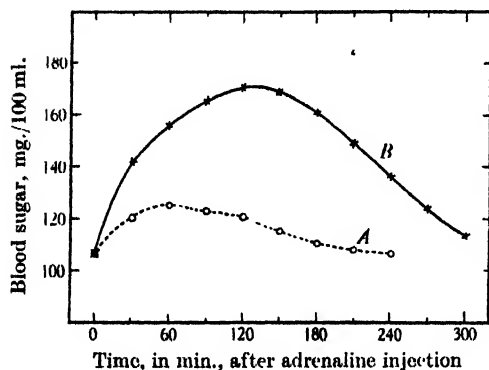
Fig. 8. The influence of treatment with the glycotropic substance on the hyperglycaemic action of subcutaneously administered adrenaline in the rabbit. *Curve A.* The average blood sugar response to the subcutaneous administration of 20 μg. of adrenaline in 10 normal rabbits which had fasted 21 hr. *Curve B.* The average blood sugar response to the same subcutaneous dose of adrenaline in the same rabbits after each had received two injections of the glycotropic substance during the preliminary fast.
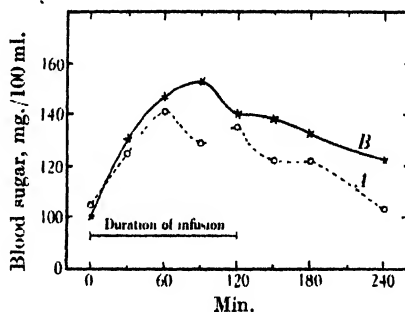
Fig. 9. The influence of treatment with the glycotropic substance on the hyperglycaemic response to the intravenous infusion of adrenaline in the rabbit. *Curve A.* The average blood sugar response to the intravenous infusion of adrenaline at the rate of 0·05 μg./kg. body weight/min., for 2 hr., in 7 normal rabbits which had fasted for 21 hr. *Curve B.* The average blood sugar response to the intravenous infusion of adrenaline at the same rate in the same rabbits after each had received two injections of the glycotropic substance during the preliminary fast.

As stated above, Corkill *et al.* [1933] and Cope & Marks [1934] found that the hyperglycaemic effect of subcutaneously administered adrenaline was greatly diminished in hypophysectomized rabbits. Russell & Cori [1937] have recently shown that the effect of the administration of adrenaline on the blood sugar level of the anaesthetized hypophysectomized rat was somewhat diminished when the adrenaline was given subcutaneously, but was normal when the adrenaline was given by slow intravenous infusion. These authors suggest that hypophysectomy diminishes the rate of absorption of adrenaline from the subcutaneous tissues of the rat. A comparison of the hyperglycaemic action in the normal rabbit treated with glycotropic substance, of adrenaline administered subcutaneously and by slow intravenous infusion, was therefore of interest.

Fig. 9 gives the average result for seven experiments in which a normal rabbit which had fasted 21 hr. received a slow intravenous infusion of adrenaline for 120 min. at the rate of 0·05 μg./min./kg. body weight (curve A), and the average response to adrenaline infused for the same time and at the same rate in the same group, when each animal had received two injections of Prolactin (1), containing altogether 1 unit of glycotropic activity, during the preliminary fast (Curve B).

The slight differences between corresponding points on these two curves are not statistically significant. The results show, therefore, that although the hyperglycaemic response to subcutaneously administered adrenaline is enhanced in the rabbit treated with the glycotropic substance, the response to the slow intravenous infusion of adrenaline was, under the conditions of our experiments, unchanged within the limits of experimental error.

As hypophysectomy diminishes the rate of absorption from the subcutaneous tissues [Russell & Cori, 1937; Heinbecker & Weichselbaum, 1937], it is possible that the administration of pituitary extracts also influences the rate of subcutaneous absorption. According to Heinbecker [1937] removal of the pituitary gland increases the action of adrenaline on the smooth muscle of the cat, and Heinbecker & Weichselbaum [1937] suggest that in the hypophysectomized animal adrenaline would cause an accentuated contraction of the blood vessels at the site of the subcutaneously injected adrenaline, with a consequent diminution in the rate of absorption. These authors consider that this diminution may account for the decreased hyperglycaemic action of subcutaneously administered adrenaline. If the injection of our pituitary extracts has an action on the response of smooth muscle to adrenaline, which is the converse of that of hypophysectomy, then the administration of glycotropic pituitary preparations might be expected to increase the rate of absorption of adrenaline from the subcutaneous tissues. The curves in Fig. 8 show that the action of subcutaneous adrenaline on the blood sugar is more prolonged in the pituitary-injected animals, and it would seem impossible to explain this effect by a more rapid absorption of adrenaline from the subcutaneous tissues. At present there seems to be no obvious explanation as to why the hyperglycaemic effect of subcutaneously injected adrenaline is so greatly enhanced in the pituitary-treated animals. The slight increase in response to the intravenous infusion of adrenaline in the pituitary-treated animals, although not statistically significant on the basis of the number of experiments carried out, may nevertheless indicate a slightly greater sensitivity to the hyperglycaemic action of adrenaline which has reached the blood stream, as the result of treatment with the glycotropic substance. As the treated rabbits have more glycogen in their livers at the end of the preliminary fasting period than do control animals [Young, 1937, 2] a slight increase in sensitivity to the hyperglycaemic action of adrenaline would be explicable on this basis alone, without recourse to a hypothesis that the liver glycogen becomes more easily mobilized under the influence of the pituitary extract [cf. Cope & Marks, 1934].

It should be recorded in this connexion that in our experience rabbits treated with the glycotropic substance are very liable to exhibit hyperglycaemia as the result of a slight emotional stimulus. No special investigation has been made on this point, and it is impossible to say whether or not observations of this kind would be found consistently in all rabbits, but it has become increasingly clear during a long series of experiments with the glycotropic substance, that in general, careful handling of the pituitary-treated animals is required if the blood sugar is to remain at its normal level. It is conceivable that the hyperglycaemic action of nerve stimuli is enhanced in these animals. The action of sympathin on the blood sugar has recently been investigated by Bodo & Benaglia [1938], but the action of pituitary extracts on the degree of hyperglycaemia following nerve stimulation in animals with denervated adrenal glands, has apparently not yet been investigated.

Collip [1938] finds that the injection of his pituitary anti-insulin substance inhibits the hyperglycaemic response to injected adrenaline. In our experiments there has been no indication of an inhibition of adrenaline hyperglycaemia in the pituitary-treated rabbits.

DISCUSSION

The results of the present investigation confirm the assumption that the pituitary gland contains a substance, the injection of which does not significantly alter the blood sugar of the fasting rabbit under the conditions of our experiments, but induces a complete insensitivity to the hypoglycaemic action of insulin. It was previously found that the daily injection of large doses of this "anti-insulin" substance into normal dogs did not induce glycosuria or ketonuria and that the "diabetogenic" and "anti-insulin" principles were therefore not identical [Young, 1936, 1, 2; 1938].

The name "glycotropic factor" was suggested as a convenient one for the pituitary substance which antagonizes the hypoglycaemic action of insulin without itself inducing a hyperglycaemia [Young, 1936, 2]. The term "glycotropic" was not intended to imply any specific mechanism of action of the "anti-insulin" factor, although at the time when the name was suggested it was believed that one action of the glycotropic substance was a facilitation of hepatic glycogenolysis, particularly the glycogenolytic action of adrenaline. Even at that time it was realized that this view was not altogether satisfactory [Young, 1936, 1], and the results since obtained do not support it. At present the mechanism whereby the anti-insulin action is exerted is not clear (see below), but until more is known about the nature and mechanism of action of the principle concerned, it is proposed to retain the name "glycotropic" factor or substance to describe it.

The identity of the glycotropic substance

The results of the present investigation differentiate the glycotropic substance from prolactin (pigeon crop gland-stimulating substance), the thyrotropic and gonadotropic hormones, and the oxytocic and pressor substances of the pituitary gland. Tissues other than the pituitary gland have failed to yield active extracts. So far there is no evidence as to whether or not the glycotropic substance is identical with the adrenotropic, ketogenic or glycostatic [Russell & Bennett, 1936] pituitary substances, or with the pituitary substance which increases the fat content of the liver [Best & Campbell, 1936].

According to a recent preliminary communication by Collip [1938], the pituitary melanophore-expanding substance, the ketogenic and anti-insulin substances, and a pituitary substance which antagonizes the hyperglycaemic action of adrenaline, are all identical. The single principle responsible for all these effects is believed to be produced in the pars intermedia of the pituitary gland. The full evidence for the belief that only one principle is responsible for so many different effects is not yet available. The results of the present investigation throw no light on the question whether or not the glycotropic substance is identical with the substance or substances responsible for the effects considered by Collip, and a discussion of this question is therefore not profitable at present.

In our experience loss of activity always occurs when preparations of the glycotropic substance are dried, and, as shown in Tables III and IV, Prolactin (1), which is an undried extract, contains a much higher proportion of glycotropic activity than the other preparations used. Our extracts containing the glycotropic substance can be dialyzed in cellophane bags (Visking) without undergoing appreciable loss of activity, but Collip [1938] finds that a substance capable of neutralizing the hypoglycaemic action of insulin will pass through a cellophane or collodion membrane, if the solution has been previously adjusted to pH 10 and heated on a boiling water bath for one hour. Further investigations on the

stability of the glycotropic substance towards heating in alkaline solution are required before the relation between the results of the present investigation and Collip's interesting findings is clear.

The most active preparations of the glycotropic substance were obtained from anterior lobe which had been carefully dissected from posterior lobe tissue, so that these preparations were virtually free from oxytocic activity (Table II). It is therefore safe to assume that the glycotropic substance is present in either the pars anterior or the pars intermedia, both of which may be present in the anterior lobe as obtained by our method of dissection. Collip [1938] believes that his anti-insulin substance is formed in the pars intermedia, but at present no evidence is available to determine whether or not this is true for the glycotropic substance.

The mechanism of action of the glycotropic substance

Cope & Marks [1934] found that in hypophysectomized rabbits the blood sugar often fell to a very low level and the animals exhibited symptoms of hypoglycaemia in spite of the presence of ample reserves of liver glycogen. They also found that the hyperglycaemic action of subcutaneously injected adrenaline was decreased in hypophysectomized rabbits and increased in rabbits treated with suitable anterior lobe extracts. These authors therefore put forward the attractive hypothesis that the anterior lobe of the pituitary gland contains a principle which renders the glycogen stores of the liver more susceptible to the mobilizing action of adrenaline [cf. also Young, 1936, 1]. More recently Cope [1937] has found that in the fasting hypophysectomized young rabbit, the blood sugar level is maintained until the liver glycogen stores are depleted; in the absence of liver glycogen there is a rapid fall of the blood sugar to convulsive levels. As the hyperglycaemic action of intravenously infused adrenaline is probably unaffected both by hypophysectomy [in the rat—Russell & Cori, 1937] and by treatment with pituitary extracts [in the rabbit—present communication] the hypothesis of Cope & Marks is not supported. This hypothesis is also not in agreement with the observations of Himsworth & Scott [1938, 2] who found that the glycotropic substance was active in abolishing the hypoglycaemic action of insulin administered to adrenalectomized rabbits.

The injection of insulin lowers the blood sugar level of the intact animal because the production of sugar by the liver is inhibited [Cori et al. 1923; Cori, 1931], and because the processes of glycogen formation in the muscles and sugar oxidation in the peripheral tissues generally are stimulated [Best et al. 1926]. Marks [1936] found, with eviscerated cats receiving glucose intravenously, that pre-treatment with suitable anterior pituitary extracts inhibited the action of insulin in increasing the muscle glycogen level, while Himsworth & Scott [1938, 2] found that the accelerating action of insulin on the spontaneous fall of the blood sugar level which normally occurs in hepatectomized rabbits, is lacking if the rabbits are treated with a preparation of the glycotropic substance before hepatectomy. We may therefore assume that, in intact rabbits, treatment with the glycotropic substance inhibits the increase in the peripheral absorption of sugar which normally follows the administration of insulin. The complete abolition of insulin hypoglycaemia in the pituitary-treated animals shown in Fig. 2 is explicable on the basis that the action of insulin in inhibiting the production of sugar in the liver has also been abolished under the influence of the pituitary extract. The form of curve illustrated in Fig. 1 is more difficult to explain. The simplest assumption is that in the livers of these animals the glycogenolytic action of adrenaline, secreted in response to the fall of blood sugar, has been greatly exaggerated by treatment with the pituitary extract, so that the

initial fall of blood sugar causes an excessive outpouring of sugar from the liver. However, as we have seen above, there is no reason to suppose that the glycogenolytic action of adrenaline in the liver is greatly increased under the influence of our pituitary extracts. It is possible, of course, that in the liver the effectiveness of glycogenolytic nervous stimuli is increased under the influence of the glycotropic substance, and that such stimuli normally play a part in raising the blood sugar to its normal level after insulin hypoglycaemia. Definite evidence on this point is lacking, however, but it may be mentioned that Cori & Cori [1927] have found that the fall of liver glycogen which normally follows the injection of a small dose of insulin into a fasting mouse is just as marked in adrenalectomized animals as it is in normal mice. This suggests the possibility that a stimulus other than that of adrenaline secreted from the adrenal glands is acting on the liver during insulin hypoglycaemia.

Rabbits treated with pituitary extracts containing the glycotropic substance have more liver and muscle glycogen at the end of a short period of fasting than do normal animals. One would expect that the presence of a large amount of glycogen in the liver would modify the extent of the fall of blood sugar level after insulin administration, but the result illustrated in Fig. 4 shows that raising the liver glycogen by this means to over 75 % above its normal fasting value had no very great influence on the hypoglycaemic response to the injection of 2 units of crystalline insulin. This suggests that the liver glycogen level cannot by itself be the controlling factor in the production of insensitivity to the hypoglycaemic action of insulin by treatment with our pituitary extracts. We must conclude, therefore, that until further results are available, the simplest assumption is that of a direct antagonism, both in the liver and peripherally, between the action of the glycotropic substance and that of insulin.

SUMMARY

1. The glycotropic (anti-insulin) principle of the pituitary gland is not identical with any of the following pituitary substances: prolactin (i.e. the pituitary substance capable of stimulating growth of the pigeon crop gland), thyrotropic hormone, gonadotropic hormone, oxytocic and vasopressor substances.

2. Preparations of the glycotropic substance containing no detectable prolactin, and preparations of prolactin containing no detectable glycotropic substance, have been obtained.

3. In rabbits which are rendered insensitive to the hypoglycaemic action of 2 units of insulin by treatment with the glycotropic substance, the hyperglycaemic action of subcutaneously injected adrenaline is substantially increased, but the effect of intravenously infused adrenaline is not significantly affected. In such rabbits sugar tolerance is slightly diminished.

4. The glycotropic substance apparently does not antagonize the hypoglycaemic action of insulin by facilitating the glycogenolytic action in the liver of adrenaline secreted by the adrenal glands. It may directly antagonize the action of insulin both in the liver and in the peripheral tissues.

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CCI. SERUM PROTEINS IN NORMAL AND PATHOLOGICAL CONDITIONS

I. THE BLOOD SERUM OF NORMAL ANIMALS

II. HUMAN BLOOD SERUM AND PATHOLOGICAL BODY FLUIDS

III. HORSE SERUM STUDIED BY MEANS OF THE PRECIPITIN REACTION

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ALTHOUGH Loeb [1922] has shown that the properties of proteins need not be described vaguely as complex colloidal phenomena but that ordinary physico-chemical principles provide a basis for adequate explanation, and although chemical methods have been successfully applied in demonstrating that other biologically important substances are compounds of definite chemical constitution, nevertheless, in the case of proteins their complexity has led to the tendency to place them in a class apart. Blood serum, for example, is sometimes regarded as a single complex protein incapable of being separated into fractions of definite chemical composition, and any fractions that are obtained are regarded as artefacts [Block, 1934]. An alternative suggestion is that serum consists of innumerable fractions differing only slightly from one another or which are even interconvertible.

These views, which would render the chemical investigation of proteins of very limited value, have not been accepted in this or preceding communications but, as a working hypothesis, it has been assumed that the serum proteins consist of a limited number of chemical individuals of reproducible properties. The objects of the investigation, therefore, have been to prepare fractions as pure as possible, to seek means of identifying individual proteins and to obtain criteria of purity of each.

Although the albumin fraction of serum has been generally regarded as consisting essentially of a single protein, nevertheless as early as 1884 Halliburton, on the basis of coagulation temperatures, suggested that albumin did not consist of a single homogeneous fraction. The same conclusion was reached by Sørensen [1930] on the basis of solubility measurements and by Hewitt [1934] as the result of carbohydrate determinations and it was eventually possible [1936; 1937; 1938] to separate from the albumin fraction of serum three distinct proteins, crystalbumin, seroglycoid and globoglycoid, which are distinguishable by biological, chemical and physical methods.

Chemical and physical evidence suggests that serum globulin is a mixture of different proteins [Hewitt, 1927; 1934; 1938; Lustig & Haas, 1931; Tiselius, 1937; Stenhagen, 1938] and this communication describes attempts to carry further the identification of chemically individual proteins in the blood serum of various animals and in various body fluids of human patients.

I. THE BLOOD SERUM OF NORMAL ANIMALS

The isolation and properties of crystalbumin and seroglycoid, the main constituents of the albumin fraction, have been described previously and attention in this section is directed mainly to the globulin fractions.

In a former communication [1938] two euglobulins, designated I and II respectively, were described in the case of horse serum. Euglobulins of similar characteristics have now been obtained from human, ox and rabbit sera. Euglobulin-1 separates out first when globulin fractions are dialysed in neutral solution; it is obtained in the form of a white precipitate requiring a fairly high concentration of salt to render it soluble. Euglobulin-II is precipitated when the solution from which euglobulin-I had been precipitated is either brought to pH 6 or else is subjected to prolonged dialysis against distilled water to remove traces of salt. Euglobulin-II is obtained in the form of a semi-liquid precipitate frequently greenish-blue in colour and it is soluble in the presence of traces of salts. In many respects it resembles the "viscous protein" of Doladilhe [1936] and its composition will be dealt with in later sections of this paper.

The water-soluble globulins or pseudoglobulins may be separated into fractions of varying properties, the fraction most readily precipitated by $(NH_4)_2SO_4$, Na_2SO_4 or NaCl having the lowest carbohydrate content [Hewitt, 1934; 1938]. The precipitin reaction of this fraction has been studied by Kendall [1937] who calls it water-soluble euglobulin or α-globulin. Since euglobulins are insoluble in water and since the latter name may be confused with α-globulin of Tiselius [1937], it is considered preferable to call the fraction *pseudo-globulin-A*.

The main bulk of the pseudoglobulin-A fraction is precipitated by one-third saturation with $(NH_4)_2SO_4$, or by saturation with NaCl, but considerable amounts remain unprecipitated and nearly all the globulin fractions so far examined contain a certain amount of this protein. It is precipitated by comparatively low concentrations (about 20 %) of alcohol in the absence of salt but contrary to common expectation higher concentrations of alcohol are necessary in order to effect precipitation in the presence of small amounts of salt.

The main pseudoglobulin fraction is not precipitable by one-third saturated $(NH_4)_2SO_4$ but is precipitated when the $(NH_4)_2SO_4$ concentration is raised to 50 % saturation. After dialysis to remove salts and euglobulins, the pseudoglobulin remaining in solution, as will be shown later, appears to consist of at least three different proteins, including pseudoglobulin-A and globoglycoid.

Globoglycoid is a globulin fraction first obtained from the albumin fraction of horse serum [Hewitt, 1938]. After removal of the main bulk of the seroglycoid the crystalbumin frequently contains considerable amounts of globoglycoid which may be separated by neutralizing the solution and precipitating with 55 % saturated $(NH_4)_2SO_4$ solution. It is now found that the serum globulin fraction precipitated between the limits 45 and 55 % saturation with $(NH_4)_2SO_4$ is rich in globoglycoid, and a similar fraction was obtained by precipitation between the limits 20 and 22 % Na_2SO_4 [Hewitt, 1934]. Removal of other albumin and globulin fractions from globoglycoid is difficult and the difficulties are enhanced by the accumulation of lipins in the fraction. In the case of human, ox and rabbit serum globoglycoids the solutions are frequently milky in appearance whilst even with horse serum globoglycoid the protein is difficult to centrifuge down owing to the presence of fatty matter. As will be shown later globoglycoid is of importance from the point of view of euglobulin formation.

It is not easy to make quantitative determinations of the distribution of the various proteins in serum by fractionation processes, since the repeated reprecipitations necessary to effect purification result in large and unpredictable losses. In addition, as will be shown later, figures for euglobulin depend upon a variety of experimental conditions so that figures in the following table must be regarded as approximate only, but they serve to show the differences between the serum protein distributions of different animal species.

Table I. *Distribution of serum proteins in blood of different animals*

Results are given in g. per 100 g. total protein.

	Horse	Ox ·	Rabbit	Human
Euglobulin	12	17	8	17
Pseudoglobulin	46	28	16	22
Crystalbumin	33	47	66	53
Seroglycoid	9	8	10	8

In the serum of a horse immunized with diphtheria toxoid the crystalbumin content fell from 33 to 13 % of the total with a corresponding increase in the globulin fractions. Pathological changes in human serum are dealt with in a later section.

The value of the carbohydrate content in distinguishing protein fractions has been indicated in previous papers [1934; 1936; 1937: 1938]. Crystalbumin has been shown to be carbohydrate-free and seroglycoid to contain about 7 % of galactose-mannose [1936; 1937]. In the case of rabbit serum as mentioned previously [1937] the solubility relations of the constituents of the albumin fraction are such that separation is sometimes difficult as mixtures of proteins tend to be precipitated together. Some specimens of seroglycoid have a carbohydrate content appreciably greater than 7 % owing to the presence of mucoid which is readily separated by reprecipitation processes.

It is, however, the carbohydrate contents of the globulin fractions with which we are chiefly concerned at present and some of the results obtained are summarized in Table II. The modification of the Sørensen & Haugaard [1933] method previously described was used except that for colorimetric comparison, in place of the step-photometer, a photoelectric Spekker absorptiometer with filter 5 was used for some of the measurements.

Table II. *Carbohydrate contents of globulin fractions*

g. of galactose-mannose per 100 g. of protein.

Fraction	Horse	Ox	Rabbit	Human
Pseudoglobulin-A	1·4	1·4	1·5	1·5
Main pseudoglobulins	2·4	3·6	2·5	3·4
Globoglycoid	5·6	4·9	4·1	5·4
Euglobulin-I	1·8	2·1	2·6	2·8
Euglobulin-II	3·6	3·4	3·2	4·0

Just as in the cases of crystalbumin and seroglycoid the carbohydrate contents of the corresponding globulin fractions of the different animal sera resemble one another but there are several points of additional interest.

In each case pseudoglobulin-A has the lowest carbohydrate content, about 1·4 %, and preliminary experiments indicate that further purification processes, although leading to considerable losses of the material, may reduce the carbohydrate content even further. The main pseudoglobulin fractions of human and

ox serum have a higher carbohydrate content than those of horse serum probably owing to the smaller proportion present of pseudoglobulin-A with its low carbohydrate content.

Composition of euglobulin-II

Some specimens of pseudoglobulin-A and the main pseudoglobulin fraction when mixed together produce a precipitate and Kendall [1937] suggests that euglobulin is a mixture of pseudoglobulins. The matter is, however, a rather complicated one.

As mentioned above and in a previous communication [1938] there appear to be two distinct euglobulins and it is euglobulin-II of which the properties and behaviour suggest that it may be composed of mixed water-soluble pseudoglobulins. Furthermore, experiments have so far failed to produce euglobulin precipitates when horse, ox or rabbit serum protein fractions were mixed and there has been success only when human serum fractions participate in the reaction, although as will be seen later there is evidence that horse serum euglobulin-II contains pseudoglobulin-A.

The next observation was that pseudoglobulin-A gave the heaviest precipitation of euglobulin when it was mixed not with the main pseudoglobulin fraction but with globoglycoid.

Proteins from the sera of different animals were mixed and it was found possible to obtain a euglobulin-II of mixed origin, the components being derived from different animal species. The results are summarized in Table III.

Table III. *Euglobulin-II precipitates from serum globulin fractions of different animal species*

+ indicates precipitation, 0 no precipitation.

Globoglycoid from	Pseudoglobulin-A from			
	Human	Horse	Ox	Rabbit
Human	+	0	0	0
Horse	+	0	0	0
Ox	+	0	0	0
Rabbit	+	0	0	0

It will be seen that in every case the pseudoglobulin-A constituent must be of human origin, whilst the globoglycoid may be derived from human, horse, ox or rabbit serum and yet produce a precipitate. The globoglycoid fraction is rich in lipins which have been considered to be concerned in euglobulin formation and it was decided to investigate the precipitating powers of globoglycoid freed from lipins. For the removal of lipins a more rapid modification of a method previously described [Hewitt, 1927] was used.

60 ml. of globoglycoid solution cooled to 0° were added gradually to 360 ml. of alcohol-ether (7 : 3) mixture kept at −15°. After being allowed to stand for an hour the precipitate was centrifuged down in chilled centrifuge buckets. The supernatant fluid was decanted off and the solid was suspended in 400 ml. of alcohol-ether mixture at −15°. After centrifuging the solid was washed twice in a similar manner with chilled ether which had been distilled over sodium metal. The whole process up to this stage had occupied less than 2 hr. The solid was now transferred to a Soxhlet apparatus and extracted with ether, sodium metal being present in the extraction flask to remove any water or alcohol present. After 2 hr. extraction, fresh ether and sodium were added and the extraction was continued for a further period of 3 hr. The solid was then dried

in vacuo over sulphuric acid and was finally obtained in the form of a fine white powder readily soluble in water.

It was found that this lipin-free globoglycoid still precipitated with human pseudoglobulin-A indicating that the lipin content of the globoglycoid was not an essential factor for the precipitation.

Some quantitative experiments on the amount of precipitate obtained under different experimental conditions are summarized in Table IV.

Table IV. *Amount of euglobulin precipitated by mixing human pseudo-globulin-A with various proteins*

Precipitate in mg. obtained from 10 mg. of mixed proteins.

Proportion of pseudoglobulin-A present %	Human serum pseudoglobulin (main fraction)		Serum globoglycoid from			
	pH 6	pH 7	Human	Horse	Rabbit	Ox
85	—	—	4·0	·	1·2	—
75	—	—	5·0	3·4	1·9	—
67	—	2·6	6·0	—	2·4	4·1
60	3·1	2·0	6·0	2·4	2·9	—
50	2·7	1·1	—	2·0		·
40	2·0	—	—	—	—	—

Precipitation is greater at pH 6 than at pH 7 as would be expected from the properties of euglobulin II already mentioned. The precipitate obtained with globoglycoid was greater than with the main pseudoglobulin fraction, suggesting that the globoglycoid present in the main pseudoglobulin fraction was the active precipitant.

Further discussion of the data described is postponed until consideration of the evidence given in later sections.

II. HUMAN BLOOD SERUM AND PATHOLOGICAL BODY FLUIDS

Human sera, urine, ascitic fluids and pleural effusions have been subjected to fractionation processes by the method already outlined with a view to detecting any considerable abnormality in the distribution of the various protein fractions.

The results of some of the fractionations are summarized in Table V. Dr Alston and Dr S. H. Robinson of the Archway Hospital Group Laboratory very kindly sent the specimens investigated.

Table V. *Protein fractions obtained from various human body fluids*

g. per 1 l. fluid.

Fraction	Normal serum	Heart failure ascitic fluid	Liver Cirrhosis ascitic fluid	Chronic parenchymatous nephritis					
				Serum	Urine	Ascitic fluid	Ascitic fluid	Pleural fluid	Pleural fluid
Pseudoglobulin-A	5·4	0·4	1·9	2·6	—	0·5	0·2	1·6	0·4
Pseudoglobulin (main fraction)	5·1	0·8	1·7	5·7	1·2	0·9	0·6	1·7	1·5
Euglobulins	8·4	1·1	1·3	5·0	1·2	1·2	0·8	2·6	1·7
Crystalbumin	25·3	8·0	3·4	3·8	1·7	1·1	1·0	3·2	2·0
Seroglycoid	3·6	2·3	2·2	5·4	1·8	0·8	0·7	1·2	0·4

The number of specimens examined is limited and it is inevitable that material is lost during fractionation processes, but despite its limitations the information obtained presents several points of interest, especially with regard to the various body fluids obtained from the case of chronic parenchymatous nephritis. All these specimens were obtained from the same patient, a man aged 51 with albuminuria and generalized oedema of a recurrent type; the blood urea concentration varied from 0·06 to 0·11 %.

The low albumin : globulin ratio in such cases of nephritis is well-known but we now know that the albumin fraction includes seroglycoid and globoglycoid in addition to crystalbumin. It is interesting, therefore, to consider the crystal-bumin concentration apart from the other components of the albumin fraction. Thus the albumin : globulin ratios of the normal and nephritic sera in the table were 1·4 and 0·8 respectively, whilst the ratios of crystalbumin : "other proteins" were 0·8 and 0·2 respectively. The difference is, therefore, much more striking when the crystalbumin alone is considered instead of the whole albumin fraction. The explanation of this is discussed later.

There is some evidence of a decreased proportion of pseudoglobulin-A in the globulin fraction of the nephritic specimens as has been indicated by the precipitin tests of Kendall [1937].

The properties so far examined of the various fractions were similar irrespective of whether their origin was serum, urine, ascitic fluid or pleural effusion. The carbohydrate content of the pseudoglobulin-A fractions approximated to 1·5 % and that of the globoglycoids to 4 or 5 % although the quantity of material available precluded careful purification. The carbohydrate contents of the main pseudoglobulin fractions varied from 2·3 to 4·4 % according to the proportions present of the various proteins comprising the fraction. Pseudo-globulin-A and globoglycoid derived from different body fluids, when mixed, yielded a precipitate of euglobulin-II just as in the case of proteins obtained from serum.

The high carbohydrate content (16·4 %) of a crude specimen of seroglycoid from nephritic ascitic fluid suggested the presence of the mucoid investigated by Zanetti [1897] and Bywaters [1909], and fractionation with $(NH_4)_2SO_4$ resulted in the separation of two fractions, one mainly seroglycoid containing 8·5 % of galactose-mannose and the other, probably mucoid containing 20 % of carbohydrate.

III. HORSE SERUM STUDIED BY MEANS OF
THE PRECIPITIN REACTION

The difficulty of obtaining pure antigens and the corresponding specific antisera and the ease with which biological tests are influenced by slight variations in experimental conditions, must necessarily set limits to the value of the precipitin reaction, and the accuracy attainable in chemical analysis is not reached. Nevertheless, precipitin tests are of great value for the qualitative examination of the homogeneity of protein fractions. Results of some quantitative significance may be obtained by observation of the first tube in a graded series of dilutions to exhibit flocculation [Dean & Webb, 1926], and, in conjunction with this method, by determining the nitrogen content of the floccules precipitated under appropriate conditions [Heidelberger & Kendall, 1932].

The importance of the route and mode of inoculation in the preparation of antisera is apparently greater than is generally appreciated and this will be discussed in a later section.

The antisera used were prepared by twice-weekly intravenous injections into rabbits of suspensions of alum-precipitated proteins over a period of 3 or 4 weeks, the course of injections being repeated if necessary. Antisera to four different fractions were investigated and they are described below.

Antipseudoglobulin-A serum reacted only with pseudoglobulin-A and on other zone of precipitation was observed.

Antigloboglycoid serum reacted principally with globoglycoid but a subsidiary zone of precipitation was observed and this was traced to the presence of antibody to crystalbumin, which was removed by absorption in the optimal proportion with pure crystalbumin.

Anticrystalbumin and *antiseroglycoid sera* reacted only with the homologous antigens.

Titrations of the antigens with the homologous antisera are summarized in Table VI.

Table VI. *Precipitin reactions of antigens with their homologous antisera*

Antisera were diluted 1 in 20 and mixed with equal volumes of antigen of dilutions given in table.

Antigen	100	300	1000	3000	10,000	30,000	100,000
Pseudoglobulin-A	–	–	–		+ + +	+ +	+
Globoglycoid	–	+	+ + +	+ +	+	–	–
Crystalbumin	–	–	–	–	+ + I	I I·	+
Seroglycoid	–	–	–	–	+ + ⊥	+ ⊥	+

Having thus obtained four specific sera each reacting with one of the protein constituents of serum, it became possible to investigate the composition of various sera and serum protein fractions. The method of carrying out the precipitin tests was to mix equal volumes of antiserum, diluted 1 in 20, with graded dilutions of the protein solution ranging from 1 % down to one-hundred thousandth or less of this concentration, and to observe the tubes at intervals for the first appearance of opalescence and flocculation.

Tested in this way good specimens of pseudoglobulin-A and crystalbumin behaved as homogeneous proteins, seroglycoid contained small amounts of crystalbumin whilst globoglycoid was found to contain small amounts of pseudoglobulin-A and crystalbumin. The main pseudoglobulin fraction also contained pseudoglobulin-A and it appears that nearly all globulin fractions contain this protein in greater or less amount.

Suitable proportions were found by preliminary tests in graded dilutions, and quantitative determinations were then carried out [Heidelberger & Kendall, 1932] by mixing the antisera with various fractions, keeping the proportions present in the region of antibody excess. After standing overnight in the ice-chest the precipitates were centrifuged down, washed with saline and the nitrogen contents determined by the micro-Kjeldahl method, the protein : nitrogen factor of 6·45 being used throughout in the calculations.

´ The antisera used were first standardized by determining the amount of precipitate produced by known amounts of the appropriate protein and plotting the curve connecting these quantities (Fig. 1). The amount of any of the four

proteins present in a fraction may be ascertained by determining the amount of precipitate obtained with the appropriate antiserum and reading off from the curve the corresponding quantity of antigen.
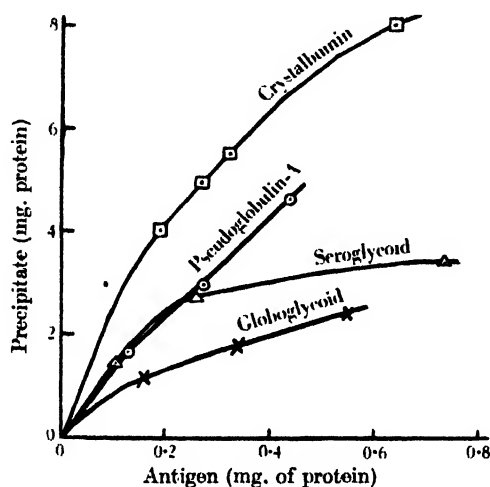
Fig. 1. Amount of protein precipitated on adding various horse serum proteins to the corresponding rabbit antisera.

The results are summarized in Tables VII, VIII, IX and X.

Table VII. *Pseudoglobulin-A antiserum precipitin reactions*

Specimen examined	Amount of specimen taken	Protein precipitated mg.	Pseudoglobulin-A present (calculated) mg.
Pseudoglobulin-A	0·13 mg.	1·68	—
,,	0·27 mg.	2·99	—
,,	0·44 mg.	4·61	—
Horse serum 471	0·02 ml.	5·02	0·490
489	0·02 ml.	5·68	0·565
478	0·0033 ml.	3·37	0·310
Globoglycoid	2·2 mg.	2·95	0·270
Total globulins	0·91 mg.	5·85	0·585
Euglobulin-II	0·45 mg.	3·08	0·283
Pseudoglobulin (main fraction)	0·41 mg.	3·16	0·290

Table VIII. *Globoglycoid antiserum precipitin reactions*

Specimen examined	Amount of specimen taken	Protein precipitated mg.	Globoglycoid present (calculated) mg.
Globoglycoid	0·16 mg.	1·17	—
,,	0·34 mg.	1·79	—
,,	0·55 mg.	2·39	—
Horse serum 471	0·10 ml.	2·07	0·46
489	0·10 ml.	2·08	0·47
478	0·10 ml.	2·36	0·59
Pseudoglobulin (main fraction)	3·50 mg.	1·68	0·34

Table IX. *Crystalbumin antiserum precipitin reactions*

Specimen examined	Amount of specimen taken	Protein precipitated mg.	Crystalbumin present (calculated) mg.
Crystalbumin	0·19 mg.	4·02	—
,,	0·27 mg.	4·96	—
.,	0·32 mg.	5·52	—
.,	0·64 mg.	8·05	—
Horse serum 471	0·02 ml.	5·78	0·345
489	0·02 ml.	6·30	0·400
478	0·07 ml.	8·05	0·640
Globoglycoid	4·40 mg.	5·24	0·290

Table X. *Seroglycoid antiserum precipitin reactions*

Specimen examined	Amount of specimen taken	Protein precipitated mg.	Seroglycoid present (calculated) mg.
Seroglycoid	0·11 mg.	1·45	—
,,	0·26 mg.	2·71	—
,,	0·74 mg.	3·43	—
Horse serum 471	0·10 ml.	3·03	0·35
489	0·02 ml.	1·45	0·11
478	0·10 ml.	2·71	0·26

Some interesting conclusions can be drawn from the tables. The main pseudoglobulin fraction of the horse serum examined was found to consist of 70 % of pseudoglobulin-A and 10 % of globoglycoid, leaving 20 % to be accounted for in the form of some other pseudoglobulin. Human serum pseudoglobulin contains only 30 % of pseudoglobulin-A according to Kendall [1937]. The higher proportion of pseudoglobulin-A in horse serum pseudoglobulin probably accounts for the lower carbohydrate content of horse serum fractions compared with human serum fractions, as mentioned previously.

Globoglycoid was first separated from the albumin fraction [Hewitt, 1938], but precipitin tests now show that the main pseudoglobulin fraction also contains about 10 % of globoglycoid. This is supported by the fact that the pseudoglobulin fraction precipitated between the limits 45 and 55 % saturation with $(NH_4)_2SO_4$ has the properties of globoglycoid.

Euglobulin-II from horse serum was found to contain 63 % of pseudoglobulin-A; this may be compared with the figures 48–65 % obtained for human euglobulin [Kendall, 1937].

The difficulties of purifying globoglycoid have already been discussed and they are illustrated by precipitin reaction results which indicated that the specimen of globoglycoid examined contained 6 % of crystalbumin and 12 % of pseudoglobulin-A.

Results of the precipitin analysis of whole horse serum are of particular interest and are summarized in Table XI.

Table XI. *Analysis of horse serum by precipitin reactions*

Results are given in g. of protein per 100 ml. of serum.

Fraction	Normal horse No. 471	Normal horse No. 489	Diphtheria antitoxin horse No. 478
Pseudoglobulin-A	2·45	2·83	9·30
Globoglycoid	0·46	0·47	0·59
Crystalbumin	1·73	2·00	0·91
Seroglycoid	0·35	0·55	0·26
Other proteins	2·02	1·82	1·34

The diphtheria antitoxin horse (No. 478) had received a series of injections of formalized diphtheria toxin, the antitoxin content of the serum had risen to 500 A.U. per 1 ml. and was investigated because the protein content had increased considerably. It will be seen that the pseudoglobulin-A content had increased threefold, whilst the crystalbumin content had fallen to less than half the normal value. It is possible that the fall in crystalbumin content might be a compensatory mechanism to balance the increased osmotic pressure of the serum caused by the increase in globulin concentration.

The figures given for "other proteins" present in serum are obtained by difference and are approximate only. These other proteins probably include euglobulin-I, and a pseudoglobulin. This pseudoglobulin is difficult to isolate owing to the difficulties of removing pseudoglobulin-A, etc.

The antisera were used to study the absorption of proteins from the peritoneal cavity. A rabbit was injected intraperitoneally with 10 ml. of horse serum. At intervals blood was withdrawn from the ear vein and the amounts of the various horse serum proteins in the rabbit's blood serum were followed by carrying out precipitin tests, using the optimal proportions method [Dean & Webb, 1926]; the considerable quantities of antisera required for the Heidelberger & Kendall [1932] method were not available. By comparison of these results with those obtained using purified antigens of known protein content it was possible to calculate the approximate quantity of each protein present.

Table XII. *Amount of horse serum proteins present in the blood serum of a rabbit injected intraperitoneally with 10 ml. of horse serum*

mg. protein per 100 ml. rabbit serum.

Time hours	Pseudoglobulin-A	Globoglycoid	Crystalbumin	Seroglycoid
0·25	25	10	20	6
1	80	–	140	—
4	100	100	210	60
24	80	—	180	—
96	80	–	140	–
336	25	—	7	—

The highest concentration of the foreign proteins was reached after 1–4 hr. and diminished quite slowly.

The serum and cerebrospinal fluid of several patients who had received intravenous injections of horse serum containing meningococcus antitoxin were also examined by the optimal proportions technique. Dr Burtenshaw of the Southern Group Laboratory kindly sent these specimens. The horse serum proteins were readily detectable in the presence of human serum proteins, and the highest concentrations recorded were: in the serum, 800 mg. of pseudoglobulin-A and 150 mg. of crystalbumin: and in the cerebrospinal fluid, 9 mg. of pseudoglobulin-A and 4 mg. of crystalbumin. The results refer to the quantity present in 100 ml.

DISCUSSION

It is becoming increasingly clear that serum cannot be regarded as a mixture of only two proteins, globulin and albumin. The albumin fraction has been separated into three different proteins, crystalbumin, seroglycoid, and globoglycoid, of distinctive properties [Hewitt, 1934; 1936; 1937; 1938]. Dr Philpot kindly examined a specimen of seroglycoid in the ultracentrifuge and reported that the sedimentation constant was not appreciably different from that of crystalbumin. Albumin prepared by electrophoresis [Tiselius, 1937] still contains

large amounts of seroglycoid as shown by its high carbohydrate content. Thus although both by chemical and precipitin tests crystalbumin and seroglycoid have been shown to be distinct and different proteins, their properties are such that certain physical methods may fail to differentiate them. Kekwick [1938] obtained an albumin fraction which by ultracentrifugal and electrophoretic methods appeared to be homogeneous, yet which by precipitin tests [Gell & Yuill, 1938] was found to be a mixture.

Previous work has revealed the presence in serum of globulins of different properties [Hewitt, 1927; 1934; 1938]. Pseudoglobulins may be obtained by fractionation with $(NH_4)_2SO_4$, Na_2SO_4 or NaCl, with carbohydrate contents ranging from 1·4 to 6·0 % (calculated as galactose-mannose). The pseudoglobulin of lowest carbohydrate content, about 1·4 %, is obtained by several reprecipitations with one-third saturated $(NH_4)_2SO_4$ solution. As the result of a study of precipitin reactions of a similar fraction obtained from human serum Kendall [1937] concluded that it was a single protein. The alternative names suggested for this protein both have disadvantages and without making any assumption as to its identity the protein is therefore described as *pseudoglobulin-A*. Closely similar pseudoglobulin-A fractions have been obtained from human, horse, ox and rabbit sera. There is, however, a difference between pseudoglobulin-A obtained from human sera and that from the sera of other animals, and this difference will be described in the next few paragraphs.

Under certain conditions, when pseudoglobulin-A and the main pseudoglobulin fraction are mixed a precipitate is obtained [Kendall, 1937]. The semifluid consistency of this precipitate resembles that of euglobulin-II, and precipitin tests also indicate that pseudoglobulin-A is a constituent of euglobulin-II. It has been found that pseudoglobulin-A gives a heavier precipitate of euglobulin with globoglycoid than with the main pseudoglobulin fraction. This, and the fact that the main pseudoglobulin fraction has been found to contain globoglycoid makes it seem likely that this latter protein is the active precipitant. Since much lipin accumulates in the globoglycoid fraction it was thought possible that this was an essential factor but it was found that globoglycoid freed from lipin still acted as a precipitant.

Mixing pseudoglobulin-A and globoglycoid from human sera (or other body fluids) resulted in precipitation of euglobulin but this did not occur when the appropriate fractions of the sera of other animal species were mixed. Furthermore, mixture of human serum globoglycoid with pseudoglobulin-A from horse, ox and rabbit sera gave no precipitate. On the other hand pseudoglobulin-A from human serum gave a precipitate when it was mixed with human, horse, ox or rabbit serum globoglycoid. It is of some interest that it is possible to prepare a euglobulin of mixed origin derived from two different animal species.

Antisera have been prepared in rabbits reacting specifically with pseudoglobulin-A, globoglycoid, crystalbumin and seroglycoid from horse serum. With these antisera it has been possible, using a quantitative precipitin technique, to obtain analytical figures for the composition of various fractions and for horse serum itself. It is now possible to quote figures for the bulk of the serum protein in terms, not of arbitrary fractions, but of proteins which although difficult to purify nevertheless may be regarded as chemical entities.

The tentative figures obtained for normal horse serum were: pseudoglobulin-A, 2·6 %; globoglycoid, 0·5 %; crystalbumin, 1·9 %; seroglycoid, 0·5 %; other proteins, 1·9 %. The figure for "other proteins" is arrived at by difference and is not accurately determined but it probably includes a new pseudoglobulin fraction and euglobulin-I as well as traces of mucoid.

Precipitin analysis of the serum of a horse after a series of injections of formalized diphtheria toxin showed that the pseudoglobulin-A fraction had increased threefold to 9·3 % whilst the crystalbumin content had fallen to 0·9 %, less than half the normal value. The reason for the increase in pseudoglobulin-A is obscure since diphtheria antitoxin appears not to be associated with this fraction [Hewitt, 1934; 1938].

Fractionation of the proteins present in human serum and pathological body fluids revealed that, as in the case of urinary and serum albumin [1927; 1929], the proteins from different sources could not be distinguished from each other. Thus pseudoglobulin-A and globoglycoid when mixed gave a precipitate of euglobulin regardless of whether the proteins originated in serum, urine, ascitic fluid or pleural effusions. The carbohydrate contents of the proteins from different sources also were similar.

It was found that there was a very marked fall in the crystalbumin content of the serum of a case of chronic parenchymatous nephritis but the seroglycoid constituent of the albumin fraction was not diminished in amount. Seroglycoid was present in considerable amounts in nephritic urine, ascitic fluid and pleural effusions but yet the concentration in the serum had not fallen as it had in the case of crystalbumin. It would seem, therefore, that mechanical loss through the kidney did not of itself account for the diminished amount of crystalbumin in the blood stream, and it appears that regeneration of crystalbumin must proceed more slowly than that of seroglycoid or the globulins. Perhaps it is significant in this connexion that crystalbumin, alone among the serum proteins so far isolated, is chemically different in not containing polysaccharide in the molecule. The slow regeneration of albumin compared with globulins after plasmaphoresis was observed by Kerr et al. [1918]. Liver damage appears to delay the resynthesis of albumin [Foley et al. 1937]. As mentioned previously the explanation is lacking of the large increase in pseudoglobulin-A content of the serum of horses injected with diphtheria toxin and indeed the whole problem of serum synthesis is obscure.

The species specificity of the various serum proteins made it possible to study the appearance of foreign proteins in the blood stream or cerebrospinal fluid after intraperitoneal or intravenous injection of foreign protein. A rabbit received an intraperitoneal injection of horse serum. Within a quarter of an hour after the injection horse serum pseudoglobulin-A, globoglycoid, crystalbumin and seroglycoid could be detected in the rabbit's blood by means of precipitin tests. Disappearance of the foreign proteins from the rabbit's blood was slow, and traces were still detectable after a fortnight. On the other hand, it is reported that after intravenous injection of egg albumin the foreign protein disappeared from the blood stream very rapidly, 94 % having gone after 2 hr. and 99·6 % after 24 hr. [Kenton, 1938].

After intravenous injection of horse serum containing meningococcus antitoxin into patients, traces of pseudoglobulin-A and crystalbumin were detected in the cerebrospinal fluid.

The question of the relative antigenicities of different proteins is complicated; there is, for example, considerable divergence in the literature concerning the antigenicity of serum albumin. That albumin is non-antigenic is maintained by some workers [Nolf, 1900: Landsteiner & Calvo, 1902; Ruppel et al. 1923] whilst others find it yields a specific antibody [Michaelis, 1902: Hektoen & Welker, 1924; Kabat & Heidelberger, 1937; Gell & Yuill, 1938]. The confusion was in some measure due to the fact that the serum albumin fraction contains at least three different antigenic proteins, crystalbumin, seroglycoid and globoglycoid,

each giving rise to a separate antibody, but there appears to be an additional source of confusion. It was reported previously that purified crystalbumin was only feebly antigenic when injected intraperitoneally even in large doses, whilst serum globulins, under these conditions are potent antigens [1937]. When injected intravenously crystalbumin is antigenic [Kabat & Heidelberger, 1937; Gell & Yuill, 1938] especially when the injected material is in the form of an alum suspension. The use of alum in enhancing antigenic activity was reported by Glenny et al. [1926]. Not only does the antigenic activity increase in the presence of alum but the risks of anaphylaxis after intravenous injection, previously reported [Hewitt, 1937], appear to be minimized. It is important, therefore, in attempting to evaluate the relative antigenicities of different proteins to take into account the route and method of injecting the antigens.

SUMMARY

1. Attempts have been continued to separate serum into chemically distinct homogeneous individual proteins.

2. Pseudoglobulin-A, globoglycoid, crystalbumin and seroglycoid have been detected in human, horse, ox and rabbit sera.

3. Conditions have been studied under which mixing of pseudoglobulin-A and globoglycoid produces a precipitate of euglobulin-II.

4. In a nephritic patient the protein fractions were similar whether derived from serum, urine, ascitic fluid or pleural effusion and they could not be distinguished from normal serum proteins.

5. Both seroglycoid and crystalbumin were excreted in the urine of a nephritic patient in considerable amounts and the crystalbumin content of the serum fell to a low value, but the seroglycoid content did not fall appreciably. A different mechanism of regeneration of different serum proteins is indicated.

6. Specific precipitating sera were prepared for the four horse serum proteins, pseudoglobulin-A, globoglycoid, crystalbumin and seroglycoid. The route and method of injection of crystalbumin have marked effects on the antigenicity.

7. Approximate figures for the composition of normal horse serum in terms of four of the individual protein constituents have been obtained by means of quantitative precipitin tests. The results are: pseudoglobulin-A 2·6 %; globoglycoid 0·5 %; crystalbumin 1·9 %; seroglycoid 0·5 %; other proteins 1·9 %.

8. In the serum of a horse immunized against diphtheria toxin the pseudoglobulin-A content had increased threefold and the crystalbumin content had fallen to half its normal value.

9. A rabbit was injected intraperitoneally with horse serum and the various horse serum protein fractions were detected by precipitin tests in the rabbit's blood stream within 15 min. of the injection. The maximum concentration of foreign protein was reached after 1 to 4 hr. and fell only slowly, traces being still detectable after a fortnight's interval.

10. Traces of horse serum pseudoglobulin-A and crystalbumin were detected in the cerebrospinal fluid of patients injected intravenously with horse serum.

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CCII. THE POLYSACCHARIDE CONTENT AND REDUCING POWER OF PROTEINS AND OF THEIR DIGEST PRODUCTS

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THE carbohydrate contents of proteins are of value in differentiating individual serum proteins and in following the course of fractionation processes [Hewitt, 1934–1938]. Apart from these practical considerations, however, the presence of carbohydrate groups or polysaccharides in proteins is of theoretical interest also, from the point of view of the structure of the protein molecule.

In order to separate the polysaccharide completely from protein material very drastic chemical treatment is necessary [Levene & Mori, 1929: Rimington, 1931; Bierry, 1934; Onoe, 1936]. Whilst this may be taken as evidence that the polysaccharide forms an integral part of the protein molecule, it also raises doubts whether the constitution of the polysaccharide present in the protein molecule is necessarily the same as that of the isolated material, particularly when the yield of purified polysaccharide is very low. Any data obtainable without previous isolation of the polysaccharide are therefore of interest, so it was decided to attempt to correlate the reducing powers of various proteins with their polysaccharide contents. Below are presented data on the carbohydrate contents, glucosamine contents and reducing powers of various proteins, and of the effect of various hydrolytic and enzymic digestion processes.

EXPERIMENTAL

Carbohydrate determinations. A modification of the orcinol method [Tillmans & Philippi, 1929; Sørensen & Haugaard, 1933] was used as previously described, colorimetric comparison being made either in a Stufenphotometer, or photo-electrically using a green filter. For serum proteins results are quoted in terms of galactose-mannose and for egg proteins in terms of mannose.

Reducing powers. The reducing powers of solutions were determined by the Hagedorn & Jensen [1923] method as modified by Hanes [1929] and Hulme & Narain [1931] except that deproteinization with $ZnSO_4$ and NaOH was omitted, the protein solution being heated with the ferricyanide reagent.

Glucosamine determinations. The colorimetric methods described by Zucker-kandl & Messiner-Klebermass [1931], Elson & Morgan [1933], Nilsson [1936], and Palmer *et al.* [1937] were modified as follows for the determination of the glucosamine content of proteins. Hydrolysis was effected by heating the protein solution in a boiling water bath for 4 hr. with 2N HCl, and the solution was cooled and neutralized with NaOH. The temporary existence, during the neutral-ization, of a slightly alkaline reaction was not found deleterious, although the importance of avoiding alkalinity has been stressed by some workers. After filtration through filter paper to remove humin 1 ml. of the solution, diluted to contain 0·05–0·3 mg. glucosamine was heated with 1 ml. acetylacetone solution (2 % in 0·5N Na_2CO_3) in a boiling water bath for 15 min. Irregular results were obtained until a simple but effective form of reflux condensation was resorted to. The solutions were contained in 6 × 0·75 in. test tubes drawn out into a

constriction at the upper end and resting in this narrow neck was placed a 3×0.4 in. test tube filled with cold water. After heating, the tubes were cooled by immersion in cold water and 5 ml. of alcohol were added. The ethyl alcohol had been purified by heating with Ag_2O and NaOH and redistilled. 1 ml. of Ehrlich's reagent was added and the colour was allowed to develop for 30 min. Ehrlich's reagent was prepared by dissolving 0.8 g. purified dimethylaminobenzaldehyde in 30 ml. purified ethyl alcohol and adding 30 ml. concentrated HCl. The p-dimethylaminobenzaldehyde was purified by dissolving in concentrated HCl, diluting with water and adding sodium acetate solution. The first yellowish precipitates obtained were rejected, and further addition of sodium acetate produced a white precipitate, which yielded a satisfactory reagent. Colorimetric comparisons were carried out in a Stufenphotometer using 1 cm. cells and an S 53 filter, glucosamine solutions being examined at the same time.

Protein preparations. The blood serum proteins used were prepared as described previously. The casein, edestin, gelatin and amino-acids investigated were commercial specimens. The ovalbumin specimens used were repeatedly crystallized and the ovomucoid was obtained from the mother liquors after the successive removal of globulins, albumin and conalbumin. The ovalbumin was quite colourless but the ovomucoid was lemon yellow in colour and when pure contained no heat-coagulable protein.

Results. In Table I are summarized mean values for the glucosamine and carbohydrate contents of various proteins. The seroglycoid and globoglycoid preparations contained some crystalbumin and had correspondingly decreased carbohydrate contents.

Table I. *Galactose-mannose and glucosamine contents of proteins of blood serum and egg white*

(g. per 100 g. protein)

Protein	Galactose-mannose (G.-M.)	Glucosamine (Ga.)	Ratio $\left(\dfrac{\text{G.-M.}}{\text{Ga.}}\right)$
Seroglycoid	5.6	2.7	2.1
Crystalbumin	<0.05	<0.05	—
Globoglycoid	6.2	2.9	2.1
Pseudoglobulin	2.2	1.1	2.0
Euglobulin-II	2.7	0.8	3.5
Ovalbumin	1.8*	0.8	2.3
Ovomucoid	10.5*	9.5	1.1

* In the case of egg white proteins results are quoted in terms of mannose.

It has been shown in previous communications that purified crystalbumin contains no carbohydrate and in the present experiments it was found, as anticipated, that no glucosamine could be detected in crystalbumin hydrolysates. In the case of the other serum proteins in which the polysaccharide present is generally considered to be galactose-mannose-glucosamine the ratio of galactose-mannose content to glucosamine content should be $2:1$. With seroglycoid, globoglycoid and pseudoglobulin the deviation from this ratio does not exceed the probable experimental error; in the case of euglobulin-II the ratio appears to be high but the reason for this is not at present apparent.

In the case of ovalbumin the mannose : glucosamine ratio is in agreement with the presence of dimannose-glucosamine but with ovomucoid there are equimolecular amounts of mannose and glucosamine suggesting that the polysaccharide present is built up of mannose-glucosamine units. This will be commented upon later.

In Table II are summarized the reducing powers of various protein substances, using the Hagedorn-Jensen method; the figures quoted are in terms of glucose. The polysaccharide contents are calculated in terms of galactose-mannose-glucosamine for the serum proteins, dimannose-glucosamine for ovalbumin and mannose-glucosamine for ovomucoid.

Table II. *Polysaccharide contents and reducing powers of various proteins, etc.*

(g. carbohydrate per 100 g. protein)

Protein	Polysaccharide content	Reducing power (glucose)
Euglobulin-II	4·1	11·9
Pseudoglobulin	3·3	12·5
Globoglycoid	9·3	13·5
Crystalbumin	>0·05	17·0
Seroglycoid	8·4	14·4
Ovalbumin	2·6	9·5
Ovomucoid	20·0	23·0
Casein	0·5	7·5
Edestin	>0·1	7·5
Gelatin	0·5	0·9
Cystine	0	15·0
Glutamic acid	0	0·1
Glycine	0	0
Tryptophan	0	80·0
Tyrosine	0	100·0

It will be seen at once from Table II that the reducing power of the proteins is not dependent upon the polysaccharide content. Of the serum proteins crystalbumin has the highest reducing power although it contains no carbohydrate.

Carbohydrate, when present, probably contributes to the reducing power but it is necessary to seek some other constituent of the protein molecule, to account for a considerable portion, or in some cases all, of the reducing power. Glycine and glutamic acid were found to have no reducing power, cystine had about one-seventh of the reducing power of glucose, tyrosine had approximately the same reducing activity as dextrose and tryptophan four-fifths of the reducing power.

It is significant that gelatin has an extremely low reducing activity since it is the only protein investigated containing negligibly small amounts of cystine, tryptophan and tyrosine in addition to having a low carbohydrate content.

The Hagedorn-Jensen method for the determination of reducing power includes the liberation of free iodine at one stage and it was thought possible

Table III. *Comparison of the apparent reducing powers of proteins, etc., at room temperature and when heated with ferricyanide*

Protein	Apparent reducing power	
	When heated	At room temperature
Crystalbumin	17·0	0·5
Pseudoglobulin	12·5	0·4
Globoglycoid	13·5	1·1
Seroglycoid	14·4	0·9
Casein	7·5	1·5
Edestin	7·5	1·5
Cystine	15·0	1·0
Tryptophan	80·0	60·0
Tyrosine	100·0	45·0

that iodination of the protein might account for a substantial portion of the reducing power. In the next series of experiments, therefore, heating of the protein with the ferricyanide was omitted and the protein was mixed with the reagents in the cold and titration was conducted immediately. The figures obtained are summarized in Table III.

In the case of tryptophan, and to a less extent with tyrosine "cold" reducing power, probably associated with iodination, is very marked, but in the case of the serum proteins from 92 to 97 % of the Hagedorn-Jensen reducing power is observed only when the protein is heated with ferricyanide.

Effects of acid hydrolysis

Heating with 2N HCl for 4 hr. was sufficient to liberate the glucosamine present in the protein, but this heating had no appreciable effect on the galactose-mannose content of the protein as determined by the usual orcinol method. In most cases the effect of the heating with acid on the reducing power was also negligible. The exceptions were ovomucoid, in which the reducing power increased from 23 to 27 % (calculated as dextrose), and crystalbumin, in which the reducing power fell from 17 to 8 % during the heating with acid.

Enzymic hydrolysis

Solutions of pseudoglobulin, seroglycoid, globoglycoid and ovalbumin containing 0·125N HCl and 0·4 % pepsin were incubated at 52° for 18 hr. Acetone (from 4 to 9 vol.) was added to the cooled digestion mixture and the precipitate was centrifuged off, washed with acetone and ether and dried in vacuo. The polysaccharide contents of the dried products were 19, 19, 24 and 18 % respectively—values from three to six times higher than those of the original proteins. Products containing even higher carbohydrate contents may readily be obtained by modification of the conditions, as the following example illustrates. A globoglycoid solution of pH 1·03 containing 0·4 % pepsin was incubated at 52° for 18 hr. The digest product precipitated by 80 % acetone contained 35 % of polysaccharide (calculated as galactose-mannose-glucosamine).

The effect of experimental conditions on the digestion of pseudoglobulin containing diphtheria antitoxin is seen in Table IV. In each case 5 ml. of 3·5 % pseudoglobulin were treated with varying amounts of HCl and pepsin and the mixture was incubated for 2 hr. at 52°. After neutralizing the digestion mixture the flocculation titre was determined under standardized conditions.

Table IV. *Pepsin digestion of diphtheria antitoxin under different experimental conditions*

Digest no.	0·1 N HCl ml.	0·1 % pepsin ml.	Flocculation	
			Titre A.U./1 ml.	Time min.
1	2·0	0·5	0	—
2	1·1	0·5	250	14
3	0·9	0·5	350	14
4	0·9	0·1	350	80
5	0·7	0·5	450	22
6	0·5	0·5	500	45
7	0·5	2·0	550	14
8	0	0	600	38

It will be seen that the destruction of antitoxin appears to be due to acid and that the presence of pepsin has some protective effect as claimed by Parfentjev [1936]. The digest products in some cases actually flocculate more rapidly than the original product although chemical manipulation usually lengthens the flocculation time. The accelerated flocculation of digested material was noted by Pope & Healey [1938]. An attempt was made to purify digest product No. 3 by first removing the material precipitated by addition of 0·5 vol. acetone and then collecting and drying the product precipitated on addition of a further 1·5 vol. of acetone. When dissolved in water the flocculation time was still only 17 min. This indicates a much greater degree of stability than that of undigested antitoxin. Purification of digest No. 7 by this method was less successful, the flocculation time increasing to 43 min. The carbohydrate content of an experimental batch of digest antitoxin kindly supplied by Mr Pope was 2·5 %, a figure falling within the limits observed for normal horse serum pseudoglobulin.

DISCUSSION

It has been suggested that ovalbumin contains no polysaccharide and that the carbohydrate detected in specimens of this protein is due to the presence of ovomucoid [Levene & Mori, 1929]. The figures given by Levene & Mori for the polysaccharide content of these proteins were obtained by actual isolation, a process which would be expected to give a low yield. The results reported, 0·26 % for ovalbumin and 5·1 % for ovomucoid are, in fact, much lower than the figures, 2·7 and 21 % respectively, obtained in the present investigation. Figures of the same order as the present values have been reported by Sørensen [1934] and Onoe [1936]. Repeated crystallization has failed to reduce the carbohydrate content of ovalbumin.

In ovalbumin the ratio of mannose to glucosamine approximates to $2 : 1$ and in ovomucoid it is close to $1 : 1$. It would seem, therefore, that the polysaccharides present in the two proteins are different, in one case being composed of dimannose-glucosamine and in the other of mannose-glucosamine units. In the present work no attempt has been made to distinguish between glucosamine and its acetyl derivative. The latter is said to be present in ovomucoid by Onoe [1936]. There is thus considerable weight of evidence that ovalbumin contains polysaccharide in the molecule, unlike serum crystalbumin: the carbohydrate content of serum albumin has been traced to the presence of seroglycoid, globoglycoid, etc. [Hewitt, 1934; 1937; 1938].

It was thought possible that determination of the reducing power of proteins might be of value in gauging the amount and nature of the polysaccharides present in the protein molecule and in following the liberation of polysaccharides during the hydrolysis of proteins. The Hagedorn-Jensen method has been used for this purpose. It has been found, however, that the reducing power of proteins bears little relation to the carbohydrate content. Crystalbumin, for example, which contains no polysaccharide has the highest reducing power of all the serum proteins investigated. With each protein the reducing power was greater than could be accounted for by the carbohydrate content even assuming that the whole of the carbohydrate was free to exert its whole reducing effect.

Various amino-acids were examined and it was found that although glycine and glutamic acid did not reduce the reagents used, other amino-acids had very pronounced reducing effects. Cystine had 15 % of the reducing power of dextrose and tryptophan 80 %, whilst tyrosine had about the same reducing activity as

an equal weight of dextrose. That the reducing power of proteins is due in part to amino-acids such as cystine, tyrosine and tryptophan, receives support from the behaviour of gelatin. Gelatin was the only protein material investigated which is deficient in these particular amino-acids as well as in carbohydrate and it was the only protein with a negligibly small reducing activity. Four hours' heating with $2N$ HCl is sufficient to liberate glucosamine from serum and egg white proteins, but the reducing power is not increased by this treatment except in the case of ovomucoid where there was an increase from 23 % (calculated as glucose) before heating to 27 % after hydrolysis. Meyer et al. [1936] have attempted to show by measurements of reducing power that the bacteriolytic activity of lysozyme is due to splitting off polysaccharides from glycoproteins, but it would seem from the present experiments that the technical difficulties in following the liberation of carbohydrates from proteins by reducing power determinations are very great. In the case of crystalbumin the effect of acid hydrolysis was an unexplained fall in the reducing power to about half its original value.

By digesting serum and egg proteins with pepsin and precipitation with acetone, peptones may readily be prepared with high carbohydrate contents, the highest carbohydrate content so far observed being 35 %. Such carbohydrate-rich peptones will receive further study. In a recent short note Ogston [1938], using a commercial enzyme preparation, split off rather less than half the carbohydrate from a specimen of pseudoglobulin, leaving one-third of the protein with an unchanged ultracentrifugal sedimentation constant. Full experimental details are lacking but it is possible that one of the constituents of the pseudoglobulin fraction [Tiselius, 1937; Hewitt, 1934; 1938] may be preferentially hydrolysed.

SUMMARY

1. The glucosamine contents of pseudoglobulin, globoglycoid and seroglycoid are in rough agreement with the generally accepted view that the polysaccharide present in the proteins is composed of units having the composition galactose-mannose-glucosamine. Crystalbumin contains no glucosamine.

2. The glucosamine contents of the egg white proteins are in agreement with the view that ovomucoid contains mannose-glucosamine and that ovalbumin contains dimannose-glucosamine.

3. Evidence is presented disproving the view that the polysaccharide present in ovalbumin is due to the presence of ovomucoid.

4. The reducing power of proteins has been traced to the presence of various amino-acids such as tyrosine, tryptophan and cystine as well as to carbohydrates. .

5. The reducing power of many proteins is not increased after acid hydrolysis and the course of hydrolysis cannot therefore be followed by reducing power determinations.

6. Carbohydrate-rich peptones may readily be prepared from proteins by peptic digestion followed by acetone precipitation.

7. A few observations have been made on the effect of peptic digestion on diphtheria antitoxin globulins.

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CCIII. THE PHOTOCHEMICAL DECOMPOSITION
OF *l*-ASCORBIC ACID

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DURING the last few years evidence has been accumulating to show that the oxidation of *l*-ascorbic acid is catalysed by visible light in the presence but not in the absence of certain sensitizers. Martini & Bonsignore [1934] found that *l*-ascorbic acid reduced methylene blue to its leuco base when exposed to the light of an ordinary electric lamp. Kon & Watson [1936] observed that on exposure to visible light of short wave length (blue and violet) *l*-ascorbic acid in milk was oxidized to dehydroascorbic acid. A probable explanation for this phenomenon is found in the observation made by Hopkins [1937: 1938] that lactoflavin and to a lesser extent lumichrome could act as sensitizers in the oxidation of *l*-ascorbic acid when exposed to solar radiation.

In this communication experiments are described which show that light is capable of producing a chemical change in *l*-ascorbic acid even in the absence of sensitizers or catalysts. This photochemical action is, however, restricted to the ultraviolet region of the spectrum. It further appears that the photochemical change takes place not only with but also apparently without the intervention of oxygen. So far as cause and mechanism are concerned this reaction, therefore, differs from those observed by the above-mentioned workers.

TECHNIQUE

All the solutions were made up in phosphate buffer (*p*H 7) prepared with quartz-distilled water. This *p*H was employed in preference to *p*H 7·4, since it was more favourable to the stability of dehydroascorbic acid. The solutions (50 ml.) were exposed in glass or quartz round-bottomed flasks, according to the nature of the experiment, to a Hewittic Y 2 mercury vapour lamp at a distance of one foot. Control experiments carried out in the dark were kept at 37° since this was the temperature reached by the solutions, owing to heat emitted by the lamp, after one hour's exposure. In the anaerobic experiments the flasks were previously exhausted and filled with nitrogen three times and finally exposed in an atmosphere of this gas. Dehydroascorbic acid was determined by reducing the solutions with hydrogen sulphide at *p*H 3 after the addition of glacial acetic acid, one drop of 1 % NaCN and one drop of octyl alcohol. The hydrogen sulphide was removed in the usual way by a current of nitrogen. When sensitizers were present in solution the displacement of the hydrogen sulphide was carried out in the dark.

The behaviour of l-ascorbic acid on exposure to visible and ultraviolet light

The decomposition of *l*-ascorbic acid by the direct action of ultraviolet light can be conveniently demonstrated by following up the disappearance of *l*-ascorbic acid in solutions exposed to a mercury vapour lamp in quartz and in glass vessels

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with and without sensitizers. In the experiment to be described l-ascorbic acid solutions in phosphate buffer at pH 7 were therefore irradiated in quartz and glass flasks in the absence of a sensitizer and in glass flasks in the presence of methylene blue and of lactoflavin. In addition, a solution of l-ascorbic acid in a glass flask was kept in the dark at 37° to serve as a control. At suitable intervals samples were removed and titrated at pH 3 with indophenol, whilst a duplicate of each sample was treated with hydrogen sulphide as described above. It was found possible to determine l-ascorbic acid in the presence of methylene blue, since the latter does not interfere with the end point; a very slight excess of indophenol produces a purple coloration in the blue solution. The results in Table I show that in the absence of a sensitizer the l-ascorbic acid in the glass

Table I. *Effect of exposure of* l-*ascorbic acid to mercury vapour lamp*

mg. of l-ascorbic acid per 100 ml.

Exposure of l-ascorbic acid to mercury vapour lamp

	In the dark		In quartz		In glass		In glass in the presence of methylene blue (0·5 mg./100 ml.)		In glass in the presence of lactoflavin (0·5 mg /100 ml.)	
Time min.	Before treatment with H₂S	After treatment with H₂S	Before treatment with H₂S	After treatment with H₂S	Before treatment with H₂S	After treatment with H₂S	Before treatment with H₂S	After treatment with H₂S	Before treatment with H₂S	After treatment with H₂S
0	4·00	4·00	4·00	4·00	4·00	4·00	4·00	3·95	4·00	3·88
15	4·00	4·00	2·50	3·00	4·00	4·00	2·00	3·57	0·80	2·70
30	3·98	4·00	0·68	1·97	4·00	4·00	0·60	2·70	0·00	1·92
60	3·97	4·00	0·00	0·78	3·80	3·75	0·00	1·20	0·00	0·90

flask, i.e. the solution which was exposed to visible light only, was not decomposed to any appreciable extent after 60 min. exposure, thus resembling the l-ascorbic acid solution kept in the dark. In contradistinction to this the solution in the quartz flask which was exposed both to visible and ultraviolet light simultaneously showed a progressive diminution in the l-ascorbic acid content. As was to be expected in the presence of methylene blue or of lactoflavin the l-ascorbic acid gradually disappeared when exposed even to visible light. It will also be noted that there was a formation of dehydroascorbic acid simultaneously with the disappearance of the l-ascorbic acid in each case.

The decomposition of l-ascorbic acid by ultraviolet light under anaerobic conditions

In the preceding experiment the exposure to the ultraviolet light took place in the presence of air and, as was seen, there was a simultaneous formation of dehydroascorbic acid in spite of the absence of a sensitizer. An experiment will

Table II. *Effect of exposure of* l-*ascorbic acid to mercury vapour lamp under anaerobic conditions*

mg. of l-ascorbic acid per 100 ml.

	In the dark		Exposed to mercury vapour lamp	
Time min.	Before treatment with H₂S	After treatment with H₂S	Before treatment with H₂S	After treatment with H₂S
0	4·00	4·00	4·00	4·00
15	4·00	4·00	2·20	2·49
30	3·90	4·00	0·90	1·13
60	3·80	3·85	0·00	0·27

now be described in which the irradiation of an *l*-ascorbic acid solution took place in the absence of atmospheric oxygen. The method employed to ensure this condition has already been described. The results obtained (Table II) leave no doubt that even in the presence of such traces of oxygen as may have been left there was a photochemical decomposition of *l*-ascorbic acid. Furthermore no formation of dehydroascorbic acid could be demonstrated in this case. The slight increase in the titration after treatment with hydrogen sulphide was of a similar order to that observed in the control solution kept in the dark.

The stability of dehydroascorbic acid to ultraviolet light under anaerobic conditions

Dehydroascorbic acid was prepared immediately before use by oxidizing *l*-ascorbic acid with the theoretical amount of potassium iodate in the presence of potassium iodide and hydrochloric acid. 1 ml. of a 0·2 % solution was then added to 49 ml. of phosphate buffer (pH 7·0). The solution was exposed to the mercury vapour lamp in a quartz flask under anaerobic conditions. The determinations were carried out by titrating with indophenol the ascorbic acid formed after reduction with hydrogen sulphide. Dehydroascorbic acid is not very stable even under anaerobic conditions in the dark, most probably because of the gradual opening of the ring in the molecule [cf. Herbert *et al.* 1933], but, as will be seen from Table III, the deterioration in the exposed solution did not take place at a

Table III. *Effect of exposure of dehydroascorbic acid to mercury vapour lamp under anaerobic conditions*

mg. of *l*-ascorbic acid per 100 ml. after treatment with H_2S

Time (min.)	In the dark	Exposed to mercury vapour lamp
0	3·66	3·66
15	2·77	2·52
30	1·92	1·78
60	1·25	0·96

much higher rate than in the control solution kept in the dark. It may therefore be concluded that ultraviolet light has no very marked deleterious effect on dehydroascorbic acid in the absence of oxygen. This result is in consonance with the fact that a freshly made up solution of this compound shows no selective absorption in the ultraviolet region [Herbert *et al.* 1933].

The inability of lactoflavin to sensitize decomposition of l-*ascorbic by ultraviolet light under anaerobic conditions*

Although lactoflavin catalyses the oxidation of *l*-ascorbic acid in the light it was nevertheless desirable to ascertain whether it influenced the anaerobic decomposition of the compound by ultraviolet light. The results are given in Tables IV and V. They suggest that in the absence of oxygen lactoflavin has very little, if any, influence on the rate of the decomposition of *l*-ascorbic acid. It is true that a somewhat greater disappearance of *l*-ascorbic acid was observed when the solutions were exposed to the mercury vapour lamp in the presence than in the absence of the sensitizer. This, however, was undoubtedly due to the fact that in spite of all the precautions taken, a certain very short exposure to light and oxygen could not be avoided during the removal and titration of the samples. In the presence of oxygen lactoflavin, as was to be expected, accelerated markedly the oxidation of the *l*-ascorbic acid.

Table IV. l-*Ascorbic acid exposed to mercury vapour lamp under anaerobic conditions in the presence and absence of lactoflavin*

mg. of l-ascorbic acid per 100 ml.

| | In the dark | | | | Exposed to mercury vapour lamp | | | |
| | Without lactoflavin | | With lactoflavin (0·5 mg./100 ml.) | | Without lactoflavin | | With lactoflavin (0·5 mg./100 ml.) | |
Time min.	Before treatment with H₂S	After treatment with H₂S	Before treatment with H₂S	After treatment with H₂S	Before treatment with H₂S	After treatment with H₂S	Before treatment with H₂S	After treatment with H₂S
0	4·00	4·00	4·00	4·00	4·00	4·00	4·00	3·89
15	4·00	4·00	4·00	4·00	2·25	2·56	1·90	2·40
30	3·90	4·00	3·90	3·95	1·14	1·43	0·96	1·30
60	3·80	3·80	3·75	3·90	0·10	0·35	0·00	0·25

Table V. l-*Ascorbic acid exposed to mercury vapour lamp under aerobic conditions in the presence and in the absence of lactoflavin*

mg. of l-ascorbic acid per 100 ml.

| | In the dark | | | | Exposed to mercury vapour lamp | | | |
| | Without lactoflavin | | With lactoflavin (0·5 mg./100 ml.) | | Without lactoflavin | | With lactoflavin (0·5 mg./100 ml.) | |
Time min.	Before treatment with H₂S	After treatment with H₂S	Before treatment with H₂S	After treatment with H₂S	Before treatment with H₂S	After treatment with H₂S	Before treatment with H₂S	After treatment with H₂S
0	4·00	4·00	4·00	4·00	4·00	4·00	4·00	4·00
15	4·00	4·00	4·00	4·00	1·96	3·10	0·00	2·70
30	3·90	4·00	3·90	4·00	0·20	1·08	0·00	1·67
60	3·80	4·00	3·70	3·75	0·00	0·14	0·00	0·30

The influence of acidity on the decomposition of l-ascorbic acid by ultraviolet light

It is now well known that the atmospheric oxidation of l-ascorbic acid in the presence of metallic catalysts proceeds more slowly in acid solution than in neutral or alkaline solution and therefore a retardation in the disappearance of l-ascorbic acid when exposed to ultraviolet light in the presence of air in acid solution was to have been anticipated. It was, nevertheless, of interest to study the effect of pH on the anaerobic photochemical decomposition. l-Ascorbic acid solutions buffered at pH 3 and pH 7 were therefore exposed to ultraviolet light in the presence and in the absence of air. The results (Table VI) show that not

Table VI. *The influence of acidity upon the decomposition of* l-*ascorbic acid exposed to mercury vapour lamp*

mg. of l-ascorbic acid per 100 ml.

	Exposed to mercury vapour lamp							
	Anaerobic conditions				Aerobic conditions			
	pH 3·0		pH 7·0		pH 3·0		pH 7·0	
Time min.	treatment with H₂S	treatment with H₂S	Before treatment with H₂S	After treatment with H₂S	Before treatment with H₂S	After treatment with H₂S	Before treatment with H₂S	After treatment with H₂S
0	3·80	3·80	3·80	3·82	3·80	3·80	3·80	3·82
15	2·68	3·44	2·22	2·52	2·44	3·47	2·16	3·09
30	2·04	3·32	0·90	1·28	0·86	2·94	0·60	1·97
60	1·20	2·84	0·00	0·40	0·30	2·07	0·00	1·32

only under aerobic but also under anaerobic conditions the lower *p*H had a retarding influence on the disappearance of the *l*-ascorbic acid. Corresponding control solutions kept in the dark showed hardly any decomposition. It is of interest to note that after treatment with H_2S there was an appreciable rise in the indophenol-reducing capacity at *p*H 3 even under anaerobic conditions.

SUMMARY AND CONCLUSIONS

The results reported in this communication show that *l*-ascorbic acid, but not dehydroascorbic acid, undergoes photochemical decomposition when exposed to the light of the ultraviolet region of the spectrum without the intervention of oxygen. It may be argued that the disappearance of the *l*-ascorbic acid in the anaerobic experiments was due to the presence of the last traces of oxygen which were not removed after repeated exhaustion. The formation of dehydro-ascorbic acid owing to its comparative stability at *p*H 7 (Table III) should then have been detected, which as will be seen from Table II was not the case. Furthermore, we have observed that on exposing higher concentrations of *l*-ascorbic acid under anaerobic conditions the quantities which were decomposed were far in excess of those which could have been oxidized by any traces of oxygen which were left in solution. The following example will illustrate the point. A buffered solution (*p*H 7) of 50 ml. containing 50 mg. of *l*-ascorbic acid was exposed to the mercury vapour lamp anaerobically for 6 hr. under the above conditions: 22 mg. of the acid disappeared during this period. If the disappearance of the compound were due entirely to oxidation, 1·40 ml. of oxygen would have been necessary—a quantity which could not possibly have been present in solution even without previous exhaustion. In the presence of air, however, the oxidation of *l*-ascorbic acid is undoubtedly catalyzed by this light. Under both conditions the reactions proceed without the aid of a sensitizer. Lactoflavin which acts as a photocatalyst in the oxidation of *l*-ascorbic acid by visible light has no action on the photochemical change of this compound in the absence of oxygen. Acidity which retards the oxidation of *l*-ascorbic acid also retards, although to a lesser extent, its photochemical decomposition when oxygen is excluded. These observations taken in conjunction with the fact that the oxidation of *l*-ascorbic acid is catalyzed by visible light bring into prominence the complexity of the nature of the decomposition of *l*-ascorbic acid by light.

It may be observed in this connexion that the slightly increased decomposition of ascorbic acid recorded by Kon & Watson [1936] when milk was exposed in quartz rather than in glass vessels, was presumably due to the *direct* action of ultraviolet light. The greater part of the decomposition of the acid in the quartz vessel was, however, brought about probably in the same way as in the glass vessel, namely, as suggested by Hopkins [1938], by the action of visible light sensitized by the lactoflavin of the milk.

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CCIV. STUDIES ON CAROTENOIDS

V. GAZANIAXANTHIN

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THREE xanthophylls of the formula $C_{40}H_{56}O$ are known, of which cryptoxanthin [Kuhn & Grundmann, 1933] is related to β-carotene, rubixanthin [Kuhn & Grundmann, 1934] to γ-carotene, and lycoxanthin [Zechmeister & Cholnoky, 1936] to lycopene. In this paper we describe a fourth xanthophyll with one oxygen atom, which has been isolated from the petals of *Gazania rigens*, and which, judging from its behaviour, is related to γ-carotene and rubixanthin.

The flowers of *G. rigens* are very interesting from the biochemical standpoint since the great majority of its carotenoids belong to the γ-carotene series, which rarely occurs in other plants. The greater part of the carotenoids of this plant appears in the "carbohydrate fraction", which includes also the xanthophylls with one oxygen atom. This fraction contains four different carotenoids, three of which are present in about equal amounts, and the fourth—probably γ-carotene—only in much smaller quantities. All these four substances have practically the same absorption spectrum as γ-carotene; they can be separated chromatographically.

Chromatographic analysis of the saponified lipoid extracts of *Gazania* flowers gives an upper zone of rubixanthin, which can easily be obtained in a pure crystalline state. The second coloured zone, separated from the first by a large colourless one, contains the new xanthophyll gazaniaxanthin. It is accompanied by another unknown carotenoid with the same absorption spectrum, adsorbed closely under the zone of gazaniaxanthin, and difficult to separate from it chromatographically. This substance has not yet been obtained crystalline. Below this zone, follows a small zone of γ-carotene.

Gazaniaxanthin crystallizes from a mixture of benzene-methyl alcohol (1 : 4) in brilliant rectangular leaflets of a deep red colour, whose macroscopic and microscopic appearance is very similar to that of cryptoxanthin. The crystals contain methyl alcohol from which they can be freed only with difficulty. In this respect gazaniaxanthin is rather different from rubixanthin, which crystallizes without methyl alcohol, in red-brown needles of a much lighter colour.

Gazaniaxanthin is readily soluble in benzene and light petroleum, very sparingly soluble in methyl alcohol. During partition between light petroleum and methyl alcohol, gazaniaxanthin behaves like rubixanthin. It melts at 136–$137°$ (uncorr., in evacuated tube).

The spectral behaviour of gazaniaxanthin is very similar to that of γ-carotene and rubixanthin, the absorption maxima being slightly displaced to shorter wave-lengths.

Absorption maxima of gazaniaxanthin[1]

	mμ
In carbon disulphide	530, 495·5, 463
In light petroleum (B.P. 80°)	494, 461, 433·5
In hexane	492·5, 460, 432
In benzene	508, 476·5
In absolute ethyl alcohol	494·5, 462·5

Elementary analyses of gazaniaxanthin agree with the composition $C_{40}H_{54}O$ or $C_{40}H_{56}O$. The presence of an alcoholic hydroxyl group was demonstrated by the formation of an acetyl derivative with acetic anhydride in pyridine solution.

Gazaniaxanthin in the crystalline state binds solvents and ash tenaciously and in spite of repeated crystallizations we did not succeed in obtaining absolutely ash-free preparations. We believe that this fact is the cause of the ease with which solutions and crystals of this carotenoid undergo autoxidation, whereby it is transformed in the course of a few days even in the cold into a colourless crystalline substance.

In its chromatographic behaviour gazaniaxanthin is intermediate between rubixanthin and lycopene. A mixture of these three substances can be easily separated in the alumina column.

The elucidation of the chemical structure of gazaniaxanthin will be possible only after further investigation. Kuhn & Grundmann [1934] have demonstrated that rubixanthin is a derivative of γ-carotene containing a hydroxyl group in the β-ionone ring: it is suggested that gazaniaxanthin may be an isomeride of this having the hydroxyl group in the aliphatic side chain, like lycoxanthin and lycophyll [Zechmeister & Cholnoky, 1936]. The correctness of this supposition can be tested by a biological assay, as a substance of the constitution proposed should be active as provitamin A in contrast to rubixanthin.

EXPERIMENTAL

We are obliged to Dr Mendonça of the Botanical Institute of this University for the following notes about *Gazania* plants.

Gazania rigens, of the family of Compositae, is native to Africa. It is cultivated in Portugal in the open air, whereas in the north of Europe it can only live in hot houses. The plant, though abundant in flowers, does not fructify in Portugal, and the flowers used for our investigation are from a specimen brought to the Botanical Garden of Coimbra about a century ago, and which has since then reproduced only asexually by means of suckers.

The plant flowers from March to the end of May, only a small part flowering at a time. We collected the flowers once a week. The plant contains a latex-like sap, which is exceptionally rich in lipoids.

The petals (about 200 g. collected in a week), are separated and dried in a stream of warm air at 50° for several hours. The dry material (40 g.) is finely pulverized in a mortar, and then exhaustively extracted with hot light petroleum (B.P. 30–50°) in an atmosphere of CO_2.

The solution, on standing in the ice box, deposits crystals of free xanthophyll and a part of the resinous substance. After filtering, the solution is evaporated *in vacuo* at a low temperature until it begins to foam. The residue is dissolved in 150 ml. of hot absolute ethyl alcohol. This solution, during slow refrigeration, precipitates a considerable quantity of colourless resinous substance, which is

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[1] Determined with a Hilger prism-spectroscope and a copper sulphate-ammonia filter.

filtered off after about half an hour. It is important not to cool this alcoholic solution too much, or to let it stand for too long, as otherwise a part of the carotenoid pigments also precipitates.

The solution is filtered and 50 ml. of pure thiophen-free benzene and 5 ml. of saturated aqueous KOH are added and the homogeneous clear mixture is kept at room temperature (22°) in well filled stoppered bottles for 2 days. Then 300 ml. of light petroleum and 50 ml. of water are added and the mixture is agitated. Nearly all the colouring matter remains in the upper layer. The alkaline alcoholic layer is discarded as it contains much resinous matter and only a little xanthophyll.

The light petroleum solution is thrice extracted, each time with 50 ml. of methyl alcohol (90 %), which dissolves a quantity of xanthophyll. The solution is then washed several times with water, to remove the methyl alcohol, dried with sodium sulphate and filtered. When kept in the ice box a quantity of colourless substances (various sterols) separate, which have been obtained in a pure state.

It proved of special importance for the chromatographic separation of the carotenoids present in the light petroleum solution to choose an adsorbing substance which is not too active, as otherwise in consequence of the many colourless substances present, and the relatively small differences in adsorbing affinity of the pigment, no sufficient separation can be obtained. We have found that, though separation may be effected with calcium hydroxide, this substance is not very appropriate because of its bad filtering qualities and the indistinct formation of the zones. The best material for the separation of the carotenoids of *Gazania* flowers is alumina. We used Merck's ordinary aluminium oxyd. puriss. anhydr., which, as it was still too active, was moistened with light petroleum and kept in the air for 1 day.

The solution is filtered through a column 15 cm. high and 5 cm. in diameter, without suction, and the chromatogram is developed with a mixture of benzene-light petroleum (1 : 3). Six zones develop. The first small, deep red, zone still contains a little xanthophyll, which was recognized to be pure lutein. Then follows a deep brown-red zone 5 mm. thick, which contains rubixanthin. The third zone, 3–4 cm. thick contains only colourless substances. Then comes a violet-red zone 4 mm. thick, immediately followed by another brown-red zone of double this thickness. From the former we have isolated gazaniaxanthin, while the latter has not yet furnished a crystalline coloured substance. The isolation and crystallization of gazaniaxanthin depends essentially on the sharp separation of the upper uncoloured and the lower coloured zones.

At some distance from the fifth zone, follows a small yellow ring which passes more rapidly to the filtrate and which shows the bands of γ-carotene. A very small first part of the yellow filtrate shows absorption bands of β-carotene.

Rubixanthin. The zone of the chromatogram containing rubixanthin is eluted with light petroleum containing 1 % of methyl alcohol and evaporated *in vacuo.* The residue is dissolved in 10 ml. of hot light petroleum and kept for 2 days in the ice box. Deep red crystals and a red oil separate. The precipitate is filtered and the crystals remaining on the filter are freed from the oil by washing with cold light petroleum which contains a few ml. of absolute ethyl alcohol. The crude crystals have M.P. 145°. They are dissolved in a mixture of 15 ml. methyl alcohol and 10 ml. ether, and the solution evaporated on the water bath until crystallization begins. The solution is kept at room temperature for half an hour, then in the ice box overnight. The crystals obtained are once

more crystallized from a mixture of benzene-methyl alcohol (1 : 3). 5 mg. of fine red-brown needles are obtained with M.P. 160° (uncorr., in evacuated tube). Absorption bands in CS_2: 533·5, 498, 465 mμ; in light petroleum (B.P. 80°) 496, 461 mμ. From the mother liquors more rubixanthin of M.P. 150–153° was obtained.

Gazaniaxanthin. The zone of the chromatogram which contains the gazaniaxanthin is carefully separated from the other zones above and below and eluted with light petroleum containing 1 % of methyl alcohol. The solution is evaporated to dryness *in vacuo*. The residue solidifies within a few seconds. It is dissolved in the minimum necessary amount of hot light petroleum (5 ml. approximately) filtered and kept in the ice box. After half an hour gazaniaxanthin begins to crystallize in brilliant orange leaflets. After 20 hr. the crystals are filtered, and twice crystallized from a mixture of benzene-methyl alcohol (1 : 4). From this mixture the carotenoid crystallizes in brilliant rectangular crystals of a deep red colour with strong dichroism, and birefringence. From a mixture of light petroleum and methyl alcohol brown-red crystals of more irregular shape are obtained. Both these crystals contain methyl alcohol, of which the greater part is lost during heating *in vacuo* to 80–90°. The M.P. determined in a circular bath of sulphuric acid was found at 136–137° (uncorr., in evacuated tube).

During heating in high vacuum at 80° the carotenoid lost 0·92, 0·89 mol. of methyl alcohol.

Found (for dry and ash-free substance): C, 87·12, 87·39 %. H, 10·75, 10·73 %. Calculated for $C_{40}H_{56}O$: C, 86·89%; H, 10·22%. $C_{40}H_{54}O$: C, 87·27%; H, 9·90 %.

Gazaniaxanthin acetate. 10 mg. of gazaniaxanthin were dissolved in 23 ml. of pyridine and 2 ml. of acetic anhydride added. The mixture was kept for 1 day in a well-filled, stoppered bottle. Then 75 ml. of benzene were added and the solution shaken many times with water to remove all pyridine. The solution was dried with sodium sulphate, filtered and concentrated *in vacuo* to 3 ml. 9 ml. of absolute methyl alcohol were added and the mixture kept in the ice box. In the course of several days, the acetate crystallized. It was twice recrystallized from a mixture of benzene-methyl alcohol. The acetate tends to crystallize slowly, in the course of several days only.

Gazaniaxanthin acetate is more soluble in benzene and light petroleum than the free xanthophyll. It crystallizes from a mixture of benzene-methyl alcohol in thick star-shaped orange needles. From light petroleum-methyl alcohol fine, long, needles appear, frequently curved; M.P. 83–85° (uncorr., in evacuated tube) with strong foaming, probably losing solvent.

The absorption spectrum is identical with that of gazaniaxanthin; in CS_2: 530, 494 mμ; in benzene 508, 476, 445 mμ.

Xanthophylls. The methyl alcohol extracts obtained after saponification are, after addition of 10 ml. of light petroleum, carefully diluted with water. Crystalline xanthophyll precipitates. The ethereal solution of this crude xanthophyll gives a faint blue colour with conc. HCl, which, however, disappears after further crystallizations. The xanthophyll is recrystallized from a mixture of chloroform-light petroleum, then twice from a mixture of methyl alcohol-ether. A small portion of "leaf xanthophyll" is obtained; M.P. 204° (uncorr.). Absorption bands in CS_2: 509, 478 mμ. During chromatographic adsorption of the xanthophyll on to calcium carbonate, two zones are observed, the upper orange yellow, the lower light yellow. We have not tried to isolate the components in a pure state.

From the first zone of the chromatogram of the hydrocarbon fraction we obtained by elution with methyl alcohol a solution, from which after evaporation

in vacuo and two crystallizations from a mixture of methyl alcohol-ether, pure lutein was isolated. It crystallized in yellow needles, which, in contact with the methyl alcohol, were transformed in the course of several days into deep red crystals, which contained methyl alcohol. M.P. 195° (uncorr., in evacuated tube). Absorption bands in CS_2 508, 476, 445 mμ.

SUMMARY

1. A new xanthophyll with one oxygen atom, gazaniaxanthin, has been isolated from the flowers of *Gazania rigens*. Its physical and chemical properties are similar to those of rubixanthin.

2. In the flowers of *Gazania rigens* there occur, besides gazaniaxanthin, rubixanthin, lutein and "leaf xanthophyll", which were isolated in a pure crystalline state, and probably γ-carotene. Another carotenoid with the same absorption spectrum as γ-carotene could not be isolated in the crystalline state.

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CCV. THE CARBOHYDRATE AND FAT METABOLISM OF YEAST

V. THE SYNTHESIS OF FAT FROM ACETIC ACID: THE INFLUENCE OF METALLIC IONS ON CARBOHYDRATE AND FAT STORAGE

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WHEN yeast is suspended in oxygenated water, part of its reserve carbohydrate is transformed into lipoid: if, however, it be incubated in an oxygenated solution of sodium acetate, the amount of lipoid formed is greater and is indubitably formed at the expense of the acetate molecule [Smedley-Maclean & Hoffert, 1926; Wieland & Wille, 1935].

There appeared to be three possible ways in which this synthesis could be effected: (1) the fatty acid chains might be built up at the expense of the acetate molecules; (2) the reserve carbohydrate present in the cell might furnish some degradation product capable of reacting with the acetate molecules; (3) carbohydrate might be formed from the acetate and converted into fat.

It was therefore interesting to know whether any simple carbon compound formed by the condensation of acetic acid could be utilized for fat formation. Butyric, acetoacetic and β-hydroxybutyric acids had already been tested with negative results [Smedley-Maclean & Hoffert, 1926]; succinic acid, formed in a yield of 10 % when yeast is incubated in an oxygenated acetate solution and citric acid, formed from acetate in presence of Ba^{++} ions [Wieland & Sonderhoff, 1932], have now been tested and found to be ineffective as are also fumaric and malic acids. Acetoin, synthesized by yeast in large yields when acetaldehyde is added to a fermenting sugar solution [Neuberg & Reinfurth, 1923], was also inactive. The results of our experiments are set forth in Table I.

Yeast incubated in the solutions tested showed about the same fat content as yeast which has been incubated in oxygenated water, with three exceptions, diacetyl, methylvinyl ketone and sodium sorbate, in all of which a system of conjugated double bonds is present; this structure apparently inhibits the transformation of reserve carbohydrate into fat normally taking place when yeast is incubated in oxygenated water.

So far, therefore, it has not been possible to find evidence that any simple carbon compound containing 4–6 carbon atoms, which might be regarded as a condensation product of acetic acid, can act as an intermediate substance in the synthesis of fat.

In support of the hypothesis that fatty acids might be formed by the interaction of the acetate molecule with some degradation product of carbohydrate, experiments carried out by Stephenson & Whetham [1922] were recalled in which the addition of sodium acetate to the glucose solution in which the Timothy grass bacillus was incubated resulted in an increase of the amount of fat stored.

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Table I

Showing mg. of fat and carbohydrate contained in 10 g. yeast after incubation in an oxygenated solution of substance to be tested. The figures after incubation of 10 g. of the same sample of yeast in oxygenated Na acetate solution are given for comparison. The phosphate present in the solution was 1·27% as a mixture of sodium hydrogen phosphates pH 6·8. CH = carbohydrate; Lipoid = ether-soluble material. The methods of estimation were those used by Smedley-Maclean & Hoffert.

Substance	%	Original yeast		(1) Solution Na acetate $N/14$		(2) Solution substance		(3) Solution substance with $PO_4\equiv$	
		CH	Lipoid	CH	Lipoid	CH	Lipoid	CH	Lipoid
Na salt of:									
Citric acid	0·52	—	84	237	190	250	117	---	—
Succinic acid	0·44	—	86	250	214	377	134	310	153
	0·22	760	82	165	123	310	137	250	144
Fumaric acid	0·43	595	83	232	195	—	—	165	153
Maleic acid	0·43	—	—	---	.		---	200	145
Crotonic acid	0·32	---	—	302	203	245	109	—	—
Laevulic acid	1·00	560	86	185	207	—	.	195	128
Acetoin	0·32	637	84	237	190	—	--	167	134
	0·50	842	89	167	242		—	170	213
	0·50	750	104	180	234	--	.	192	173
	0·50	742	84	230	220	---		222	175
	0·50	645	80	192	198	—	---	192	141
	0·64	850	89	325	230	280	143	192	157
	0·78	742	82	345	185	—	--	197	151
2:3-Butylene glycol	0·66	820	87	192	230	415	128	295	153
Methylethyl ketone	0·30	—	80	--	173	..	.	-	137
Substances containing conjugated double bonds									
Diacetyl	0·65	750	91	185	196	645	84	385	81
Methylvinyl ketone	0·40	750	104	180	234	---	---	267	96
	0·40	742	84	230	220	.	.	360	77
Sodium sorbate	1·00	712	87	187	243	292	74	150	87
	1·00	802	87	212	201	337	74	187	98

The effect of adding sodium acetate to a pure glucose solution in which yeast was incubated was to produce no increase in lipoid but to bring about a considerable lowering of the carbohydrate content.

In Stephenson & Whetham's experiments the medium in which the Timothy grass bacillus was grown contained glucose, $CaCO_3$ to neutralize any free acids produced, and inorganic salts, ammonium salts furnishing a supply of nitrogen. As we were concerned only with the transformation of glucose into lipoid in the existing cells and desired to exclude the formation of new cells a nitrogen supply had to be eliminated but we proceeded to examine the effect of the addition of $CaCO_3$ to the glucose medium in which the yeast was incubated.

When $CaCO_3$ was added to the glucose solution in which the yeast was incubated the amount of lipoid fell to about two-thirds of that formed in the absence of $CaCO_3$. Addition of sodium acetate to the glucose and $CaCO_3$ medium produced, however, a slight rise in the amount of lipoid stored (cp. Table II); the carbohydrate content showed no significant alteration.

The addition of $CaCO_3$ to the glucose solution produced a distinct sedimentation of the yeast, and it seemed possible that this mechanical effect might have interfered with the formation of lipoid for which free oxygenation is essential. The replacement of the $CaCO_3$ by kieselguhr both in its original state and after washing with HCl produced fairly rapid settling of the yeast and in its

Table II

Showing the effect of $CaCO_3$ on fat and carbohydrate storage by yeast incubated in oxygenated solutions of glucose and of glucose and sodium acetate respectively. CH = carbohydrate. Figures represent mg./10 g. yeast

Original		Glucose		Glucose + CaCO_2		Glucose + Na acetate		Glucose + CaCO_3 + Na acetate	
Lipoid	CH	Lipoid	CH	Lipoid	CH	Lipoid	CH	Lipoid	CH
78	655	—	—	216	667	—	—	253	640
115	460	452	540	315	610	—	—	359	720
80	—	406	—	—	—	381	—	—	—
98	700	415	550	—	—	415	400	—	—
79	550	415	600	—	—	408	350	—	—
89	720	403	660	—	—	335	470	—	—
74	400	326	560	223	520	—	—	231	450
Mean: 88	550	402	580	251	600	385	370	281	600

original state a fall of stored lipoid similar to that produced by $CaCO_3$. After the kieselguhr had been well washed with acid, no inhibitory effect was produced on the lipoid formation: the washings contained Ca^{++} and Mg^{++} removed from the original kieselguhr. These results suggested that Ca^{++} or Mg^{++} and not the sedimentation was the inhibiting factor.

Table III

Showing mg. lipoid and carbohydrate stored in 10 g. yeast after incubation in oxygenated 2% glucose solution to which kieselguhr had been added.

		After incubation in					
Original		(a) 2% glucose solution		(b) 2% glucose solution with kieselguhr		(c) 2% glucose solution with $N/14$ Na acetate	
Lipoid	CH	Lipoid	CH	Lipoid	CH	Lipoid	CH
			(1) Unwashed				
89	670	309	745	239	862	305	—
96	672	392	580	282	560	396	457
			(2) Washed with HCl				
88	680	371	652	345	565	313	600
80	642	415	457	400	565	391	402

Table IV

Showing mg. lipoid in 10 g. yeast after suspension in oxygenated solutions of 2% glucose to which had been added (b) $CaCO_3$, (c) sodium acetate + $CaCO_3$, (d) sodium glycollate + $CaCO_3$, (e) sodium succinate + $CaCO_3$.

Original yeast Lipoid	(a) Glucose Lipoid	(b) Glucose + CaCO_3 Lipoid	(c) Glucose, CaCO_3 and $N/14$ Na acetate Lipoid	(d) Glucose, CaCO_3 and $N/14$ Na glycollate Lipoid	(e) Glucose, CaCO_3 and $N/14$ Na succinate Lipoid
102	—	227	—	381	—
79	—	329	—	397	—
78	—	216	253	276	—
84	—	195	—	256	278
115	452	315	359	—	—
74	326	223	231	—	—

The increase of lipoid produced by the addition of sodium acetate to the glucose-$CaCO_3$ medium was not specific for the acetate but was produced by the addition of other organic Na salts to the medium, and probably indicated an antagonistic effect of the Na^+ and Ca^{++} ions.

The inhibitory effects produced on lipoid storage by the addition of NaCl, $CaCl_2$ and $MgCl_2$ respectively to the glucose medium are set forth in Table V.

Table V

Showing the lipoid and carbohydrate (CH) content in mg. per 10 g. yeast after incubation in 2% glucose solution with and without the addition of chlorides. Medium oxygenated for 24 hr.

Original yeast		(a) 2% glucose		(b) 2% glucose + NaCl			(c) 2% glucose + $CaCl_2$			(d) 2% glucose + $MgCl_2$		
Lipoid	CH	Lipoid	CH	NaCl	Lipoid	CH	$CaCl_2$	Lipoid	CH	$MgCl_2$	Lipoid	CH
100	590	404	580	N/14	400	420	N/14	272	500	N/14	261	580
100	380	392	470	N/14	406	340	N/14	242	330	N/14	244	370
92	660	443	747	—	—	—	N/350	280	727	—	—	—
95	610	401	660	—	—	—	—	--	..	—	—	—
—	740	429	630	—	—	—	N/350	275	640	N/14	277	410
							N/700	333	700	N/140	298	550
							N/140	242	630	N/1400	371	580
98	740	432	610	—	—	—	N/14	228	450	—	—	—
							N/140	232	600	—	—	—
							N/1400	358	—	—	—	—

The pH of the medium at the end of these experiments was in each case approximately between 3 and 5. The variation in pH seemed to have little effect on the power of lipoid synthesis.

These experiments brought home to us the fact that the nature of the metallic ions present in the medium exercised a considerable influence on the lipoid storage. Further investigation of the comparative effects of the ions, K^+, Na^+, Ca^{++} and Mg^{++}, confirmed that both Ca^{++} and Mg^{++} exercise a very marked inhibition on the formation of lipoid material from glucose, Na^+ being almost without action; on the other hand, the presence of Na^+, Ca^{++} or Mg^{++} reduced, to some extent, the carbohydrate content.

An instance of the marked inhibitory effect of Ca^{++} and Mg^{++} was provided by some experiments in which yeast was incubated in media consisting respectively of glucose and commercial fructose solutions. The yeast incubated in the fructose solution had a very low lipoid content which showed no diminution when $CaCO_3$ was added to the medium and was much lower than the values previously recorded in similar experiments [Smedley-Maclean & Hoffert, 1923]. The sample of commercial fructose gave a good reaction for Ca and Mg but the purified fructose behaved similarly to the glucose.

The effects of K^+, Na^+, Ca^{++} and Mg^{++}, were then compared by incubating yeast in glucose solutions to which their acetates had been separately added. The results are shown in Table VI.

Here also little effect was produced on the amount of fat stored by the presence of K^+ and Na^+, but Ca^{++} and Mg^{++} produced marked diminutions, Mg^{++} apparently having the greatest influence. The inhibitory effect on lipoid storage appeared to be greater in the presence of the chlorides than of the acetates, a result consistent with the view that the inhibition brought about by Ca^{++} and Mg^{++} might be to some extent compensated by the additional lipoid formed from the acetate ions.

Table VI

Showing mg. lipoid and carbohydrate (CH) in 10 g. yeast after incubation for 24 hr. in an oxygenated solution of 2% glucose with $N/14$ acetate (0·72%).

Original yeast		Glucose 2%		Glucose 2% + K acetate		Glucose 2% + Na acetate		Glucose 2% + Ca acetate		Glucose 2% + Mg acetate	
Lipoid	CH	Lipoid	CH	Lipoid	CH	Lipoid	CH	Lipoid	CH	Lipoid	CH
80	710	398	585	—	—	381	395	311	—	—	· —
82	760	370	730	—	—	---	—	317	550	287	780
79	550	415	600	—	---	408	350	342	460	309	380
89	722	403	660	—	·--	335	470	309	520	289	570
98	700	415	550	··	—	415	400	402	370	325	370
97	530	—	550	—	--	---	—	297	440	·-	—
91	—	436	—	---	—	422	—	388	—	399	—
82	420	364	560	392	670	374	440	—	—	306	537

The carbohydrate content of the yeast was also less in the presence of Na^+, Ca^{++} and Mg^{++}, Na^+ being on the whole the most effective inhibitor of the three. Estimations of the residual sugar showed that in the presence of Ca^{++} the amount of residual sugar was higher, the utilization of the sugar having been less effective; Na^+ and Mg^{++} had no perceptible effect.

When yeast was incubated in oxygenated solutions of the pure acetates, the influence of the ions was closely similar to that produced in the glucose-acetate solutions. The presence of K^+ was most favourable for the storage of both lipoid and carbohydrate; unlike Mg^{++}, Na^+ left the lipoid content almost unaffected but like Mg^{++} it produced a decrease in the amount of carbohydrate; Ca^{++} caused a marked lowering of both lipoid and carbohydrate contents.

Incubation of yeast in oxygenated solutions of acetates ($N/14$)

Table VII

Showing mg. lipoid and carbohydrate stored in 10 g. yeast after 24 hr. incubation in acetate solutions, $N/14$.

Original yeast		After incubation in									
		(1) Water		(2) K acetate		(3) Na acetate		(4) Ca acetate		(5) Mg acetate	
Lipoid	CH	Lipoid	CH	Lipoid	CH	Lipoid	CH	Lipoid	CH	Lipoid	CH
87	700	—	—	227	343	230	248	—	—	185	262
89	843	—	—	266	263	242	168	—	—	—	—
92	825	—	—	266	232	245	230	—	—	—	—
95	523	—	—	231	260	198	180	—	—	—	—
85	650	—	—	—	—	213	240	170	250	—	·-
87	620	139	220	—	—	237	280	150	180	191	280
93	700	134	230	—	—	225	260	206	230	199	280
87	790	125	300	—	—	241	340	185	220	193	300
89	740	128	340	—	—	257	350	166	250	·-	—
89	670	111	300	—	—	240	320	181	240	—	—

The effect of increasing the concentration of acetate in the medium on the storage products was determined in solutions of the K salt. No further increase was obtained by raising the percentage of acetate from 0·29 to 1·15%.

The effects of the ions upon yeast with low and high reserves of carbohydrate were then compared. Samples of a yeast with a high carbohydrate content were suspended for 24 hr. in oxygenated water, and in oxygenated solutions of Ca and Na acetates respectively. The yeast from the oxygenated water was then

Table VIIA

Showing lipoid and carbohydrate contents in mg./10 g. yeast after incubation in K acetate solution.

	Lipoid	CH
Original yeast	92	530
Yeast after incubation in:		
0·03 % acetic acid as K salt	122	250
0·06 % ,,	158	290
0·29 % ,,	209	340
0·58 % ,,	{219	310
	{191	320
1·15 % ,,	208	270

transferred to the acetate solutions and the effects on carbohydrate and fat storage compared with those in the yeast which had been at once placed in the acetate solutions.

Table VIII

Showing lipoid content in mg. when 10 g. original yeast from brewery were incubated in oxygenated acetate solutions before and after suspension in oxygenated water. ("Run-down" yeast = yeast after suspension in water.)

	Yeast before incubation in acetate solution		Yeast after incubation in			
			Na acetate solution		Ca acetate solution	
Exp. 1	Lipoid	CH	Lipoid	CH	Lipoid	CH
Original	89	735	257	350	166	255
Run down	127	480	261	320	215	280
Difference	+38		+4		+49	
Exp. II						
Original	89	670	240	317	181	245
Run down	111	432	236	305	200	265
Difference	+22		−4		+19	

The yeast made substantially the same amount of fat whether it was at once incubated in the Na acetate solution or whether it was first incubated in oxygenated water, and then transferred to the Na acetate solution. On the other hand, the yeast which had been at once added to the Ca acetate solution had a lower fat content than the yeast transferred to the Ca acetate solution after incubation in oxygenated water. A possible explanation was that the Ca, whilst reducing the amount of lipoid formed from acetate, almost entirely inhibited the formation of fat from the reserve carbohydrate. This was, however, not confirmed since yeast incubated in solutions of $CaCl_2$, with and without $CaCO_3$, formed fat just as well as when incubated in oxygenated water. Possibly some product formed from the Ca acetate exercised an inhibitory effect.

Estimation of the residual acetate in the media. Determination of the acetate in the residual media showed that more acetate had disappeared from the Ca (30 %) than from the Na acetate solution (19·5 %). In both cases about twice as much acetate disappeared when the yeast used had already been incubated in water and contained a low carbohydrate reserve (Na acetate, 47·6 %; Ca acetate, 74·3 %).

The effect of phosphate on lipoid formation from acetate. The mean lipoid content in 8 experiments in which 10 g. yeast were incubated in 0·6 % Na acetate solution was 0·218 g.; in 16 experiments in which yeast was incubated in 0·6 % Na acetate solution containing 1·27 % phosphate (as mixed sodium

hydrogen phosphates $pH = 6.8$) the mean lipoid content was 0·222 g. The addition of phosphate to the acetate solution therefore produced no significant result.

The composition of the lipoid substance collected from a series of parallel experiments in which samples of the same yeast had been incubated in the various media, is shown in Table IX.

Table IX

Medium	Lipoid g.	Saponification value	Unsaponifiable Wt. g.	Unsaponifiable %	Fatty acids Wt. g.	Fatty acids %
(a) Original yeast	1·364	148·8	0·464	34·0	0·860	63·0
2 % glucose	2·319	160·4	0·595	25·6	1·588	68·5
0·6 % Na acetate	1·757	160·4	0·453	25·8	1·292	73·5
(b) Glucose	1·277	180·7	0·236	18·5	0·946	74·1
Glucose with Na acetate	1·206	208·9	0·119	9·9	0·921	76·4
Glucose with Mg acetate	0·963	217·8	0·087	9·6	0·768	79·7
Glucose with Ca acetate	1·092	208·7	0·099	9·1	0·880	80·6
(c) Glucose	0·727	168·0	0·198	27·3	0·501	68·9
Glucose with K acetate	0·784	155·8	0·226	28·7	0·530	67·6
Glucose with Na acetate	0·748	161·4	0·198	27·1	0·530	70·8
Glucose with Mg acetate	0·612	172·9	0·166	26·4	0·432	70·6

In Exp. (b) the addition of the acetate to the glucose medium produced a fall in the unsaponifiable from 18·9 to between 9 and 10 %: this result was not confirmed in other experiments. The addition of Na^+, Mg^{++} and Ca^{++} showed, however, a tendency to raise the proportion of fatty acids to unsaponifiable matter.

Detection of pyruvic acid and of acetaldehyde in the residual media on addition of Na^+, Ca^{++} and Mg^{++} to the glucose solution

When 2:4-dinitrophenylhydrazine reagent was added to the residual glucose and glucose-acetate solutions, a marked difference was observed in the amounts of precipitate which separated immediately at ordinary temperature.

Whereas the residual glucose solution generally remained clear or showed only a slight cloudiness, the glucose solutions to which K, Na, Ca or Mg acetates had been added gave quite copious precipitates, usually most marked in the case of the glucose-Mg acetate medium. Replacement of the acetate solutions by chlorides produced somewhat similar precipitates and the accumulation of carbonyl compounds thus appears to be promoted by the presence of K^+, Na^+, Ca^{++} and Mg^{++}.

Quantitative experiments were carried out in which 40 g. of yeast were incubated in 2 l. of an oxygenated 2 % glucose solution to which Na, Ca and Mg acetates were respectively added ($N/14$). To the resulting filtrates excess of 2:4-dinitrophenylhydrazine reagent was added and the resulting precipitates, formed after 10 min. stirring, filtered, dried at 37° and weighed. These were then extracted with Na_2CO_3 solution and the weights of acid and neutral fractions determined.

	2 % glucose	2 % glucose +Na acetate	2 % glucose +Ca acetate	2 % glucose +Mg acetate
Wt. total ppt. g.	0·22	1·93	1·82	1·71
Wt. neutral fraction	0·17	1·45	0·95	0·90
Wt. acid fraction	0·05*	0·43	0·83	0·71

* By difference.

The greater part of the neutral fraction consisted of acetaldehyde dinitro phenylhydrazone; the acid fraction consisted mainly of the pyruvic acid derivative identified by the melting point of its ethyl ester.

A number of comparative experiments were carried out in which portions of 1 g. yeast were incubated in oxygenated glucose solutions to which were added the acetate or chloride of K, Na, Ca or Mg; in some cases Mg or Ca carbonate was added to the respective chloride solution to prevent the development of acidity. The results showed considerable variation but the glucose medium to which K, Mg, Ca or Na acetate or chloride had been added always gave a precipitate with the hydrazine reagent. The glucose-acetate (K, Na and Mg) solutions, when tested with sodium nitroprusside, acetic acid and ammonia, usually developed a blue colour showing the presence of pyruvic acid in the solution. The precipitates and pyruvic acid reaction were only marked in media which had been well oxygenated. The glucose-chloride solutions, like the glucose solution, were strongly acid in contrast to the glucose-acetate solutions, the pH of which was usually 5–6.

<center>· DISCUSSION OF RESULTS</center>

<center>*Influence of metallic ions on fat and carbohydrate metabolism*</center>

Evidence already exists that the nature of the ions in the medium exercises an important influence on the fermentation process. Harden [1917] showed that washed zymin was readily activated by acetaldehyde in the presence of K but not of Na phosphate. When glucose is fermented with yeast juice, the rate of fermentation is delayed if the concentration of sodium phosphate exceeds a certain minimum. Meyerhof [1918] pointed out that NaCl and other salts depress both the rate of attainment of the maximum rate of fermentation and the maximum rate attained; Harden & Henley [1921] found that the chlorides and sulphates of Na and K diminished the maximum rate of fermentation of glucose and fructose by zymin and also the rate of attainment of this maximum, an effect not appreciably modified by the addition of acetaldehyde. The rate of decomposition of pyruvic acid was, however, uninfluenced. An inhibitory action of Na^+ on the phosphorylating coenzymes is described in recent work by Ohlmeyer & Ochoa [1937]; the activities of cozymase and adenylic acid as phosphorylating agents in a medium containing Na_2HPO_4 were 1 : 60, but after the addition of a trace of Mn^{++} to the medium the activities of both increased to 200 : 300. Cozymase was much more sensitive to the inhibitory action of Na^+ than was adenylic acid; the ratio of the concentrations of Mn^{++}, Mg^{++}, K^+ and NH_4^+, which removed the inhibitory influence of the Na^+, was 1 : 50 : 1000 : 2000. Our knowledge of the inhibitory action of Ca^{++} seems to rest chiefly on the work of Fernbach & Schoen [1913; 1914; 1920] who found that considerable quantities of pyruvic acid could be isolated from glucose fermented by various yeasts, notably *Mycolevure Duclaux* in the presence of $CaCO_3$. They ascribed the effect not only to the reaction of the medium, since when beer wort was used as the medium no pyruvic acid could be isolated even after the addition of chalk.

In Neuberg's laboratory, pyruvic acid was isolated from the action of yeast juice on Mg hexosediphosphate solution, glycerol being also formed [Kobel & Scheuer, 1930]. The addition of $MgHPO_4$, MgO or Na_2HPO_4 to a 10 % glucose solution in which fresh yeast was fermenting produced similar results. Neuberg & Kobel [1930] concluded that the phosphate ions were not concerned since MgO produced the same effect, and that Mg ions were not essential since Na_2HPO_4 produced a similar result.

The influence of metallic ions on the storage of fat and carbohydrate by yeast in oxygenated glucose media

In our experiments the addition of $CaCl_2$ or $MgCl_2$ to the oxygenated glucose solution, in which yeast was suspended, produced: (a) some diminution of carbohydrate storage, rather varying in amount, the effect increasing with the concentration of the salt (Table V); (b) marked diminution in the amount of fat stored; (c) accumulation of carbonyl compounds in the media to a varying extent.

Similar results were obtained when the acetates of Ca or Mg were added to the glucose solution, less diminution in lipoid storage being produced than by $CaCl_2$ and $MgCl_2$; possibly the acetate had contributed to the fat formation.

The addition of NaCl or Na acetate to the glucose solution markedly inhibited the carbohydrate storage, but unlike Ca^{++} or Mg^{++}, Na^+ only produced a very slight inhibition of lipoid storage. Carbonyl compounds were also detected in the medium.

The reaction of the medium did not appear to exercise a very important influence on the storage phenomena. The fat and carbohydrate contents were similar in the alkaline Na acetate-glucose and the acid NaCl-glucose media.

Ca^{++} and Mg^{++} exert a strong inhibitory influence on both fat and carbohydrate synthesis from glucose whereas Na^+ inhibits mainly the carbohydrate synthesis.

The introduction of these three ions especially of Na^+ or Mg^{++} also produced an accumulation of carbonyl compounds in the oxygenated medium. It seems possible, therefore, that this accumulation may have been connected with the diminution of power to store carbohydrate whether by inhibiting its synthesis or promoting its breakdown.

The presence of metallic ions in the solution appears to exercise an important influence on the reactions of the cell which has hitherto not been sufficiently recognized.

The formation of fat from acetates (cf. Table VII)

The influence of K^+, Na^+, Mg^{++} and Ca^{++} on the formation of fat from acetate in the absence of sugar is very similar to their effect on the formation of fat from sugar. The average increases in the fat content of a sample of yeast after incubation respectively in oxygenated solutions of $N/14$ acetates and water were:

<div align="center">

Acetate of

Water	K	Na	Mg	Ca
41%	180%	160%	118%	100%

</div>

Since the 41 % increase after incubation in water represents the fat formed from reserve carbohydrate, it is clear that fat was formed from acetates and that the inhibitory influence of Ca^{++} and Mg^{++} on the process was considerable. The carbohydrate content of the acetate yeasts was only slightly greater than that of the yeast incubated in water, and since the amount of acetate oxidized was greater in the "run-down" yeast it is probable that the acetate exercised a carbohydrate-sparing action. The level of carbohydrate in the yeast from the acetate solutions and from water was much lower than in the original yeast. We can find no convincing evidence that hydrolysable carbohydrate is formed from acetates. Wieland & Wille [1935] found in a yeast incubated in sodium acetate solution an increase of 6 % of lipoid and of 0·03 % hydrolysable carbohydrate

reckoned on the dry weight of yeast, the latter variation being probably within the limits of accuracy of the experiment. This result is in striking contrast to those obtained by incubating yeast in solutions of alcohol when large increases in the storage of hydrolysable carbohydrate took place. It is therefore possible that when fat is formed from alcohol it passes through the stage of storage carbohydrate and that no direct condensation of alcohol to fatty acid takes place.

The conclusion that fatty acid is synthesized from acetate without going through the intermediate stage of hydrolysable carbohydrate is supported by the work of Sonderhoff & Thomas [1937] on the composition of yeast grown in a solution of trideuteracetate. These workers found that under the conditions of their experiment, no exchange of light and heavy hydrogen took place between the trideuteracetate and the water in which it was dissolved. The absorptions of oxygen by yeast were practically equal in acetate or trideuteracetate solutions when tested in the Warburg-Barcroft apparatus. After shaking in the oxygenated trideuteracetate solution, the deuterium contents of the dried yeast and of its lipoid and carbohydrate were respectively determined. The acetate was proved to have taken part in the synthesis of lipoid since 14·7 % of the total lipoid hydrogen consisted of deuterium; the percentage of deuterium in the unsaponifiable material was 31·6. Since, in our experiments, incubation in an acetate solution approximately doubled the amount of lipoid originally present, and since the original lipoid did not contain deuterium, about 30 % of the newly formed lipoid hydrogen and 63 % of that of the newly formed unsaponifiable matter must have come from the trideuteracetate. Our determinations of the constituents of the lipoid formed after a brewery yeast had been incubated in Na acetate solutions, showed that the ratio of unsaponifiable to fatty acid was approximately 1 : 3, the same as in the original yeast before incubation.

If a similar relation holds in trideuteracetate solutions, approximately 20 % of the hydrogen of the newly formed fatty acid consisted of deuterium derived from the trideuteracetate. This is in agreement with our findings that the lipoid is formed by condensation of the acetate molecules.

The yeast carbohydrate (prepared by the method of Sevag & Cattaneo [1935] contained only 1·6 % of its total hydrogen as deuterium.

Although the calculation of the proportions of deuterium in the fatty acid and sterol synthesized from the trideuteracetate can only be a rough approximation, the very marked difference in the proportion of deuterated hydrogen in the unsaponifiable matter (63 %) and in the fatty acid (20 %) would seem to signify a different manner of condensation of the trideuteracetate. The comparatively small proportion of deuterium remaining in the fatty acid may signify that deuterated water is split off from the condensed molecules which would then be reduced to saturated chains by hydrogen atoms.

In the sterol synthesis, two out of three of its total hydrogen atoms must have been derived from the trideuteracetate.

The influence of phosphates on the formation of fat from acetate and from hexose

The mean lipoid content of yeast incubated in a 0·6 % solution of Na acetate was found to be unaffected by the addition of Na_2HPO_4 to the medium. Increasing the concentration of the acetate above 0·6 % diminished or failed to increase the fat content of the yeast and we could therefore only examine the influence of phosphate in dilute solutions. It is interesting that Katagiri [1926] found that

increasing the concentration of acetic acid in a glucose solution, buffered with Na acetate-acetic acid mixture, exercised an inhibitory effect on fermentation which could not be wholly ascribed to the pH.

When fat is formed from hexose, the effect of phosphate is much more obvious in concentrated than in dilute sugar solutions. Smedley-MacLean & Hoffert [1924, Table III] incubated yeast in oxygenated 4 % fructose solutions, the medium being changed every 48 hr.; their results show the close relationship that exists between the stored carbohydrate and lipoid when alkali phosphate is added to the fructose medium.

Consideration of the evidence at present available, seems perhaps to be most consistent with the view that when yeast is incubated in an oxygenated sugar solution, the formation of fat runs parallel with the storage of sugar and phosphate and is probably connected with some stage of the triosephosphate breakdown, pyruvic acid possibly providing the necessary starting material. This process is largely inhibited by the presence of Ca^{++} or Mg^{++} in the medium.

When fat is formed from acetate there is no indication that the hexosephosphate path is followed; there seems to be a mechanism by which acetate molecules can be directly condensed to form fatty acids; this process is also adversely affected by the presence of Ca^{++} or Mg^{++}. There seems to us to be no indication of the nature of the intermediate stages of this condensation. Wieland & Wille have suggested that acetic acid may be first converted into an "activated" form of succinic acid and that this may pass through oxaloacetic to pyruvic acid. This hypothesis though attractive lacks experimental basis.

Both in the formation of fat from hexose and from acetate, plentiful oxygenation is an essential condition.

SUMMARY

1. When yeast was incubated in an oxygenated solution of any of the following substances, no increase of lipoid over that produced on incubation in oxygenated water was observed: acetoin, 2:3-butyleneglycol, methylethyl ketone, sodium salts of citric, succinic, maleic, fumaric, crotonic, laevulic or gluconic acids.

2. Solutions of substances containing two conjugated double bonds, inhibited the lipoid increase normally found on incubating in oxygenated water.

3. Addition of Ca^{++} or Mg^{++} to oxygenated glucose solutions in which yeast was incubated markedly diminished the amount of lipoid normally stored. Addition of Ca^{++}, Mg^{++} or Na^{+} to oxygenated glucose solutions in which yeast was incubated lowered the carbohydrate content whether added as chloride or acetate.

4. When yeast was incubated in oxygenated acetate solutions, Ca^{++} or Mg^{++} lowered the amount of lipoid normally formed from the acetate.

5. Addition of phosphate failed to increase the amount of lipoid formed from acetate when yeast was incubated in sodium acetate solutions (0·6 %).

6. Addition of K^{+}, Na^{+}, Ca^{++} or Mg^{++} to a glucose solution in which yeast was incubated, increased the amount of carbonyl substances present in the medium. In experiments in which the acetates of these metals were added to the medium, pyruvic acid and acetaldehyde were identified.

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CCVI. FACTORS INFLUENCING BACTERIAL DEAMINATION

III. ASPARTASE II: ITS OCCURRENCE IN AND EXTRACTION FROM *BACTERIUM COLI* AND ITS ACTIVATION BY ADENOSINE AND RELATED COMPOUNDS

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THE bacterial deaminases of *Bact. coli*, previously studied, fall into two groups: the first consisting of the deaminases of glycine, *dl*-alanine and *l*-(+)glutamic acid, and the second of the deaminases of *dl*-serine and *l*-(−)aspartic acid. In these investigations deaminase activity has been measured in Q_N (μg. NH_3-N liberated per mg. dry wt. bacteria per hr.). The deaminases of the first group [Stephenson & Gale, 1937] carry out only oxidative deamination and have an activity of the order $Q_N = 30$ which remains constant throughout the growth period of the organism. Those of the second group [Gale & Stephenson, 1938] carry out both aerobic and anaerobic deaminations and have an activity of a much higher order—values of 1000 for *dl*-serine and 500 for *l*-(−)aspartic acid have been recorded—and this activity varies greatly with the age of the culture.

In the study of *dl*-serine deaminase, it was shown that the washed suspension of the organism in water lost activity on standing. Investigation of this loss and its subsequent recovery led us to postulate the existence of a diffusible coenzyme for serine deaminase. The loss of activity could be prevented by incubation with a reducing agent in the presence of phosphate, or with a trace of adenylic acid. Also, if the activity were allowed to fall, then the lost activity could be recovered by incubation for 1–2 hr. with a reducing agent and phosphate—neither being effective alone. From this evidence, we deduced that the coenzyme could exist in a reduced or oxidized form and in a phosphorylated or non-phosphorylated form, the reduced and phosphorylated form being the active substance.

Since this work was published, it has been demonstrated that the alanine oxidase preparation from kidney requires a coenzyme [Das, 1936; Warburg & Christian, 1938, 1; Straub, 1938] and that this coenzyme contains adenine, phosphate and flavin [Warburg & Christian, 1938, 2].

Harden [1901] showed that a culture of *Bact. coli* grown in glucose broth would deaminate aspartic acid with the formation of succinic acid. Quastel & Woolf [1926] showed that this reaction took place anaerobically in the absence of inhibitors but that in the presence of an inhibitor such as toluene, an equilibrium was set up corresponding to

$$\text{Aspartic acid} \rightleftharpoons \text{Fumaric acid} + NH_3,$$

the action of the toluene being to stop the later reduction of fumaric acid to succinic acid. Woolf [1929] showed that the fumarase of the organism produced malic acid from the fumaric acid so that the final equilibrium was really an

aspartic-fumaric-malic-NH_3 system. He gave the name "aspartase" to the enzyme responsible for the formation of fumaric acid from aspartic acid. These workers used anaerobic conditions for all their experiments and showed that the effect of toluene was to inhibit the reduction of fumaric acid but, since the evolution of NH_3 from aspartic acid in the initial stage of the experiment was independent of the presence of toluene, this inhibitor can have no action on the aspartase enzyme itself.

Part of this paper will be devoted to showing that there is, in *Bact. coli*, an enzyme, other than the "aspartase" of Woolf, which is responsible for the deamination of aspartic acid but which requires for its action the presence of a coenzyme which is destroyed by the method used by previous workers for the preparation of the bacterial suspension.

Methods. The strains of *Bact. coli* used, methods of growth, preparation and measurement of bacterial suspensions and the determination of NH_3 liberated have already been described [Gale & Stephenson, 1938]. Since the results obtained with the two strains do not differ significantly, only those obtained with strain I are presented here. Fig. 1 shows the effect of pH on deamination of l-($-$)aspartic acid by this strain.

The adenosine used in part of this work was obtained from Dr George Henning of Berlin-Tempelhof.
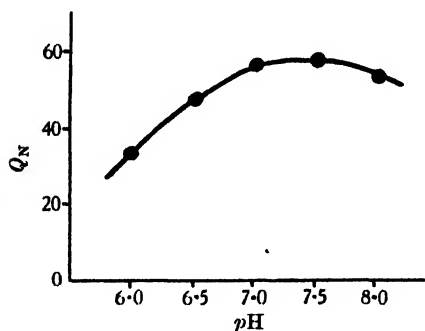
Fig. 1.
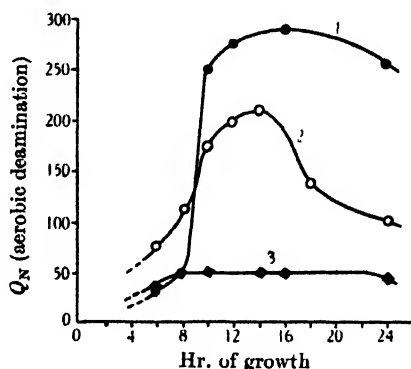
Fig. 2.

Fig. 1. Effect of pH on the rate of deamination of l-($-$)aspartic acid by *Bact. coli* (I).

Fig. 2. Variation of deaminase activity with "age" of culture and conditions of growth: 1, in N_2; 2, in air; 3, in 2 % glucose broth in N_2 (strain I).

Variation of deaminase activity with "age" of culture and conditions of growth. Fig. 2 shows the Q_N variation due to culture time and conditions of aeration. In curve 1 the culture was grown anaerobically in N_2 for varying periods; in curve 2 the growth was carried out aerobically in liquid medium in Roux bottles; in curve 3 the organism was grown in 2 % glucose broth with chalk and bubbled continuously with N_2. In the last case samples were taken at intervals with sterile precautions, the chalk removed by centrifuging for 2 min. at 3000 r.p.m. and pouring off the supernatant liquid from which the organisms could then be removed by centrifuging in the usual way. The activities in each case were determined aerobically.

The presence of glucose in the growth medium inhibits deaminase activity, a result which has been shown with all the deaminases so far studied. The low activity obtained under such conditions remains constant with age. With curve 2,

however, there is a steadily increasing activity for the first 14 hr. of growth, after which the activity falls again, reaching the initial low value after about 24 hr. When the culture is grown anaerobically, there is a sharp rise in activity between the 8th and 10th hr. of growth and the high activity then attained does not decrease appreciably up to the 24th hr. of growth.

Fig. 3 shows how this variation of activity in an anaerobic culture is related to the growth curve and also to the E_h of the medium determined with the calomel electrode. The increase in activity reaches a maximum value at about the same time as active cell division ceases and the E_h reaches a steady value. It seemed likely that the increase in activity between the 8th and 10th hr. was due to a change in the medium consequent upon the metabolic activity of the cells. To investigate this point 900 ml. of liquid medium were inoculated with a 12 hr.
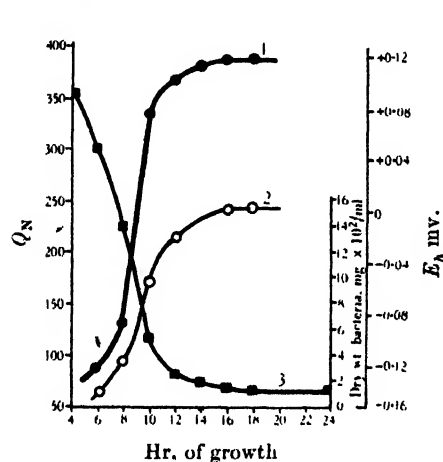
Fig. 3.   Fig. 4.

Fig. 3. 1, Variation of deaminase activity; 2, growth in dry wt.; 3, E_h of medium, with "age" of culture. Anaerobic culture (strain 1).
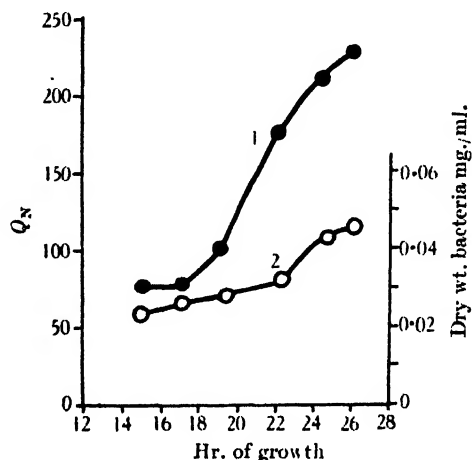
Fig. 4. 1, Variation of deaminase activity; 2, growth curve for culture grown aerobically in inorganic medium + 2% lactate + 2% aspartate (strain 1).

culture of the organism and incubated anaerobically for 10 hr., by which time the activity of the culture was approaching its maximum. The greater part of the organism was then centrifuged off and the medium sterilized by passage through a Seitz filter. This semi-exhausted medium was then re-inoculated with a 12 hr. culture, bubbled with N_2 and incubated. A turbidity was visible after 5 hr. and the organisms were spun off after 6 hr. The results of three determinations of the activity are set out in Table I. The earliest obtainable culture in the semi-

Table I. *Activity of early cultures grown anaerobically in "semi-exhausted" medium*

Exp.	Hr. of growth	Activity Q_N	Activity of control grown in normal medium (Q_N)
142	6	376	35
143	7	296	42
153	6	378	75

In exp. 153, 1% sodium aspartate was added to the semi-exhausted medium before the 2nd inoculation.

exhausted medium has an activity equal to the maximum activity attained by a culture grown normally for 14 hr. This indicates that variation in activity is dependent upon an alteration in the chemical constitution of the growth medium.

Fig. 4 shows that a culture grown in synthetic inorganic medium [Stephenson, 1930] with the addition of 2 % Na aspartate and 2 % Na lactate, shows an increase in activity before growth stops. If the increase in activity is due to the formation in the medium of some substance, then the organism is able to synthesize that substance from the constituents of this synthetic medium and, presumably, if that substance were added to the medium in the first place, then the earliest obtainable cultures would possess maximum activity. Accordingly 6 hr. cultures have been grown in liquid medium with the following additions and, in each case, the activity of the washed suspension determined:

1 % Na fumarate; 1 % Na succinate; 1 % Na pyruvate; 0·5 % Na formate; 0·5 % alanine; 0·05 % adenosine; excess CO_2.

In no case has a significant increase of activity over the control been obtained.

Loss and recovery of activity. The greater part of the work to be presented was carried out with cultures grown anaerobically for 15 hr. and having a Q_N (aerobic) of approximately 300 after preparation of the washed suspension. As in the case of serine deaminase, it was found that the activity of the washed suspension "decayed" on standing whether at 0° or on incubation. Table II shows that if suspensions of different strengths are incubated *in vacuo* for 3 hr. at 37° there is a loss of activity which increases with dilution of the suspension, being 28 % for a suspension of 3·7 mg./ml. and 78 % for a 0·37 mg./ml. suspension.

Table II. *Effect of dilution with water during incubation for 3 hr. at 37°*

Suspension of bacteria incubated (mg./ml.)	Dilution	Suspension of bacteria for estimation (mg./ml.)	Q_N (initial = 338)	Loss of activity %
3·70	—	0·37	244	28
0·75	1/5	0·37	177	48
0·37	1/10	0·37	75	78
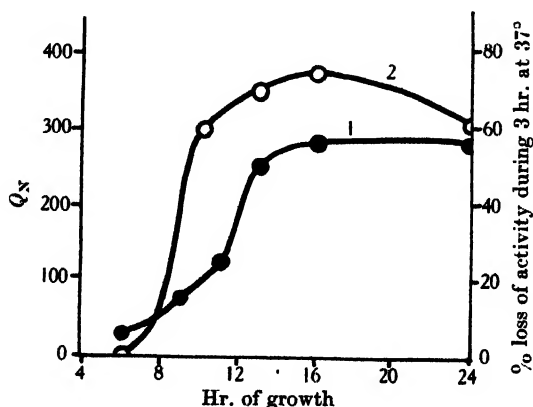
Fig. 5. Effect of "age" of (anaerobic) culture on: 1, activity (Q_N); 2, % loss of that activity during 3 hr. subsequent incubation at 37°.

With serine deaminase, it was found that the % loss of activity over any given period depended upon the "age" of the culture: an effect which was provisionally explained by differences in the permeability of the organism. Fig. 5 shows the relation between the "age" of the culture and its activity with aspartic acid (curve 1) and the % loss of activity over 3 hr. incubation *in vacuo* at 37° (curve 2). With serine deaminase, a maximum "permeability" was obtained between the 10th and 12th hr. of growth and the rate of loss of activity fell off rapidly on either side of this period: with aspartic deaminase, the rate of loss of activity remains approximately constant from the 12th to the 20th hr. of growth. The marked differences between the activity and the "permeability" curves for the two deaminases indicate that we are dealing with different systems in each case.

With serine deaminase, it was shown that the lost activity could be recovered .again by incubating the bacterial suspension for 1–2 hr. with glutathione (GSH) or more negative reducing systems in the presence of phosphate. Similar experiments have been carried out with the aspartic deaminase and the results are set out in Fig. 6. A culture was grown anaerobically for 15 hr. and the aerobic activity of the washed suspension was determined immediately after its prepara-
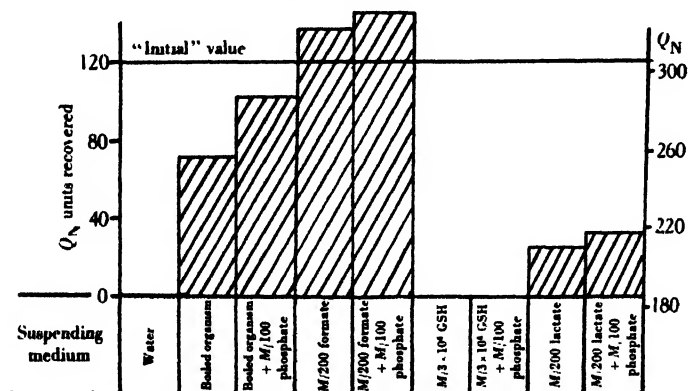
Fig. 6. Recovery of activity (strain I).

tion ("initial Q_N" = 306). The suspension was then left at 0° for 3 hr. *in vacuo*, when its activity had fallen to 186. The activity of the "decayed" suspension was then redetermined aerobically in the presence of borate buffer at pH 7·5 to which the following systems had been added: (1) water; (2) boiled bacteria in water; (3) boiled bacteria in 0·01 M phosphate buffer at pH 7·5; (4) formate, 0·005 M; (5) formate, 0·005 M, and phosphate, 0·01 M; (6) glutathione, $M/30,000$ GSH; (7) $M/30,000$ GSH and 0·01 M phosphate; (8) lactate 0·005 M; (9) 0·005 M lactate and 0·01 M phosphate (final concentrations). The results show the following marked differences from those obtained with similar experiments carried out with serine deaminase.

1. With certain additions an immediate recovery of activity is obtained without incubation.

2. GSH, with or without phosphate, has no effect either before or after incubation.

3. Boiled bacteria or formate produce an immediate reactivation with or without phosphate and, in certain cases, this reactivation proceeds to a point above the "initial" value.

Fig. 7 shows the effect of adding certain of these substances to the washed suspension immediately after its preparation. Boiled bacteria and certain reducing systems again produce an activation of the suspension. The preparation of the bacterial suspension takes about $1\frac{1}{2}$ hr. and it can be shown that there is a loss of activity during this period and that it is this lost activity which is recovered in Fig. 7. The reactivation obtained with lactate is not satisfactorily reproducible while that obtained with formate is consistent so that it seems that a reducing system is required which is rather more negative than the lactate-lactic dehydrogenase system.
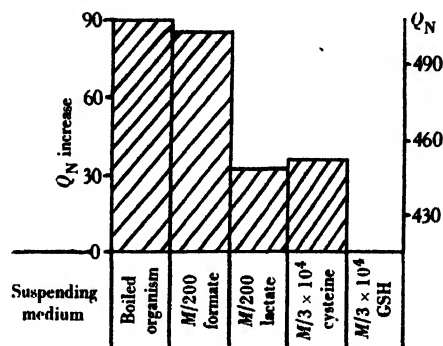
Fig. 7. Immediate activation produced by boiled organism or reducing systems.

The immediate reactivation effect of boiled bacteria suggests that they form a source of some essential factor which is lost on standing and a search for a coenzyme of l-($-$)aspartic deaminase was next undertaken.

Nature of the coenzyme. A culture was grown anaerobically for 15 hr. and the initial aerobic activity of the washed suspension determined ($Q_N = 251$). After 3 hr. at $0°$ *in vacuo* its activity had fallen to $Q_N = 84$. The activity was then redetermined in the presence of (1) boiled cell-free liver extract; (2) boiled cell-free yeast extract; (3) a suspension of boiled bacteria; (4) a cozymase preparation in solution containing approximately 0.1% coenzyme I; (5) a solution freshly prepared from a separate dry cozymase preparation containing 1 mg. of the dry material in 10 ml. The results are shown in Table III and a reactivation is obtained with the first four additions but not with the second of the cozymase preparations.

Table III. *Sources of coenzyme*

Initial activity of washed suspension		$Q_N = 251$
Activity after 3 hr. at $0°$ *in vacuo*		$Q_N = 84$

Addition to suspending medium	Resultant activity Q_N	Increase in Q_N
Boiled liver ext.	227	143
Boiled yeast ext.	161	77
Boiled bacteria	229	148
Cozymase prep. A	202	118
Cozymase prep. B	88	4

To confirm that the deaminase factor was not coenzyme I, the cozymase preparation which had proved active in the last experiment was treated in the various ways set out in Table IV. Coenzyme I is destroyed by boiling at pH 12 for 2 min. but is stable to boiling at pH 1 for 5–10 min. If, however, the

coenzyme is first reduced with $Na_2S_2O_4$ then these relations are reversed, the reduced form being stable to alkali and unstable to acid [Green & Brosteaux, 1936]. Excess $Na_2S_2O_4$ was oxidized by aeration. The form of the experiment was similar to those already described and it is obvious that the deaminase factor bears no relation to the activity of coenzyme 1 but would appear to increase slightly during alkaline hydrolysis and to decrease during acid hydrolysis. The factor is then, presumably, either an impurity in the cozymase preparation or a breakdown product of coenzyme I. The known impurities in the active cozymase preparation were then tested and are also listed in Table IV where it is seen that adenylic acid possesses a definite reactivation effect but of a rather low order.

Table IV. *Nature of active factor in cozymase prep.* A

	Initial activity of washed suspension		$Q_N - 292$		
	Activity after 3 hr. at 0 *in vacuo*		$Q_N - 206$		

Addition to suspending medium	Previous treatment. Boiled for 5 min. at pH	Coenzyme 1	Resultant activity Q_N	Q_N increase	Factor
Cozymase prep. A	—	Active	297	91	+
,,	12	Inactive	324	118	+
,,	1	Active	304	98	+
Cozymase reduced	12	Active	277	71	+
,,	1	Inactive	266	60	+
Cozymase prep. B	—	Active	200	—	—
Flavine	—	—	196	—	—
Adenylic acid (0·1 mg. ml.)	—	—	246	40	+
Nicotinamide (0·1 mg. ml.)	—	—	205	—	—
Boiled bacteria	—	—	290	84	+

It has been shown that adenine compounds are readily attacked by *Bact. coli*, the probable course being as follows [Lutwak-Mann, 1936]:

Adenylic acid →adenosine →inosine →hypoxanthine + ribose
Inosinic acid →inosine →hypoxanthine + ribose
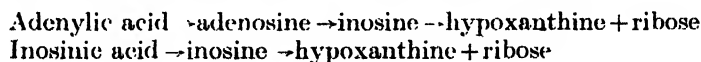
For this reason adenylic acid and its decomposition products were tested for their activating effect as follows: the activity of a washed suspension from a 15 hr. anaerobic culture was determined aerobically immediately after its preparation in the usual way with 0·03 M phosphate buffer and $M/250$ l-aspartate and then also in the presence of (1) adenylic acid; (2) inosinic acid; (3) adenosine; (4) inosine; (5) adenine; (6) hypoxanthine; (7) ribose; (8) boiled bacteria. The final concentration in all cases, with the exception of (8), was 1 mg./100 ml. The results are shown in the top portion of Fig. 8 as "initial recovery". The suspension was then allowed to stand in water at 0° for 3 hr. by which time its activity had fallen to $Q_N = 100$ and the whole experiment then repeated with this "decayed" suspension: the results obtained are shown in the lower portion and overlapping the upper portion of Fig. 8. The most effective substance is adenosine, having an activation effect greater than that produced by the boiled bacteria. It is closely followed by inosine and inosinic acid. The further breakdown products of adenosine—adenine, hypoxanthine and ribose—produce a small reactivation in the second half of the experiment but their effect is generally rather less than in the example quoted.

These results suggest that adenosine is the activating substance replaceable by inosine or inosinic acid, possibly in virtue of their interchangeability under the action of the organism [Stephenson & Trim, unpublished]. The lower but still

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marked activity of the other decomposition products of adenosine may be due to the synthesis from them of one of the three more active compounds. Until the mode of action of the adenosine etc. is explained, speculation on this point is useless. At first sight it might be thought that adenosine and inosine act as an
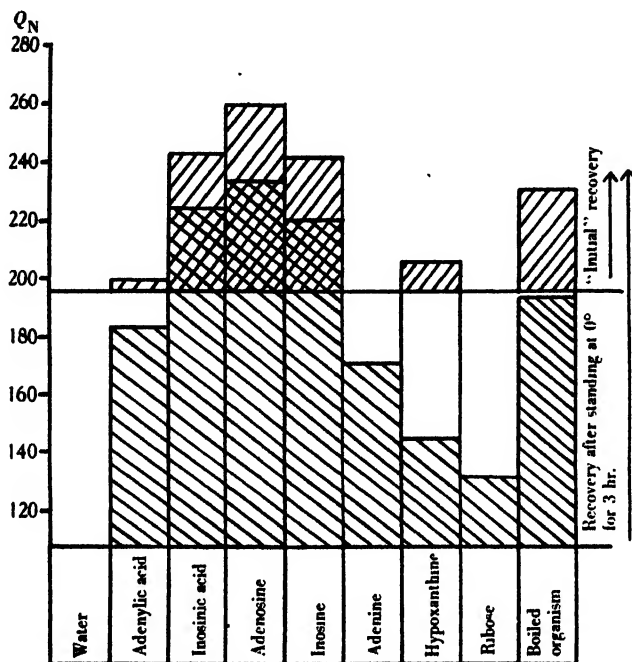
Fig. 8. Recovery in the presence of breakdown products of adenylic acid.

NH$_3$-carrier system, the inosine reacting with aspartic acid to form adenosine and the latter then being deaminated to inosine again. That this cannot be the case is seen from a consideration of the rates of deamination of aspartic acid and adenosine respectively:

Rate of deamination of l-aspartic acid:

    (a) Aerobically in presence of adenosine $Q_N = 450$

    (b) Anaerobically in presence of adenosine $Q_N = 501$

Rate of deamination of adenosine:

    (a) Aerobically $Q_N = 122$

    (b) Anaerobically $Q_N = 120$

The effect of adenosine on the deamination of 1-(−)*aspartic acid.* It was next necessary to determine whether this co-deaminase effect of adenosine would work anaerobically.

Fig. 9 shows the effect of adding an initial concentration of 0·67 mg. adenosine/ml. on the deamination and Fig. 10 the corresponding effects with one-quarter this quantity of adenosine. In the absence of adenosine there is a much slower rate of evolution of NH$_3$ anaerobically than aerobically. The effect of the addition of the adenosine is to increase the rate both aerobically and anaerobically but, whereas the aerobic rate is increased by a constant anaerobically the initial acceleration is much greater but rapidly falls off until the rate is back

to that of the control. That this is not due to the attainment of an equilibrium can be demonstrated by the addition of further adenosine, when the rate will again be temporarily accelerated.
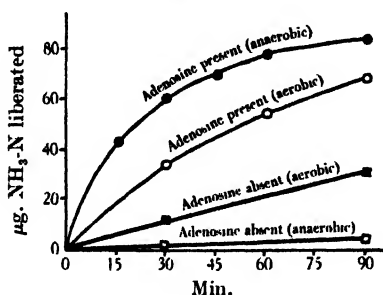
Fig. 9.
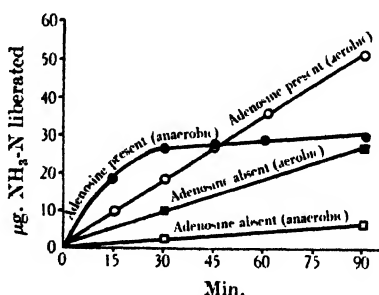
Fig. 10.

Fig. 9. Effect of adenosine (1/15 mg./ml.) on evolution of NH_3 from *l*-(−)aspartic acid in presence of *Bact. coli* (0·1 mg. dry wt./ml.).

Fig. 10. Effect of smaller concentration of adenosine (1/60 mg./ml.) on evolution of NH_3 (cf. Fig. 9).

Fig. 11 shows the result of adding increasing concentrations of adenosine upon the Q_N. Since the accelerated rate is constant aerobically (Figs. 9 and 10) we can determine a true rate under such conditions but anaerobically this is not possible and the points on the anaerobic curve of Fig. 11 represent the rate for the first 30 min. after the addition of the adenosine. We have seen from Figs. 9 and 10 that a given amount of adenosine produces a more marked increase in the anaerobic than the aerobic rate and this may mean that the total activity obtained aerobically is due to an anaerobic process. In the absence of adenosine and with freshly prepared suspensions, the aerobic rate of deamination is greater than the anaerobic but this difference in the two rates disappears on incubation of the suspension for half-an-hour. Mesrobianu [1936] has shown that *Bact. coli* contains an appreciable amount of adenosine and this difference in anaerobic and aerobic activities of fresh cultures may be due either to a labile oxidative deaminase or to the natural adenosine of the bacterium having an effect which is obvious aerobically but falls off so rapidly anaerobically (cf. Fig. 9) as not to be demonstrable.
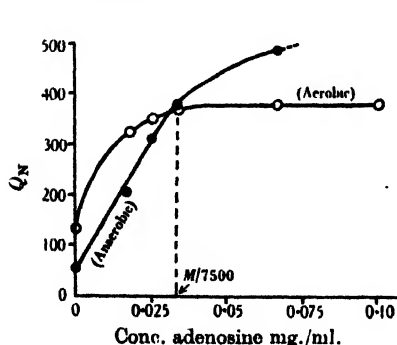
Fig. 11.
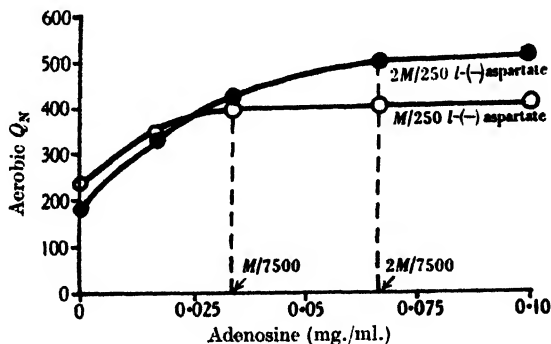
Fig. 12.

Fig. 11. Effect of adenosine concentration on rate of deamination by strain I.

Fig. 12. Variation of critical concentration of adenosine with concentration of *l*-(−)aspartate.

From Fig. 11 it is also seen that, aerobically, the rate of deamination reaches a maximum in the presence of a final concentration of adenosine greater than the critical concentration of $M/7500$. The final concentration of aspartic acid in this case is $M/250$ so that a maximum rate of deamination is obtained when there is one mol. adenosine present to every 30 mol. aspartic acid. Fig. 12 shows that if the final concentration of l-($-$)aspartic acid is doubled then the critical concentration of adenosine is also approximately doubled.

Deamination of 1-($-$)*aspartic acid by cell-free* coli-*juice.* At this point in the research the wet-crushing mill of Booth & Green [1938] became available and it was decided to test these findings with a cell-free juice obtained by crushing *Bact. coli.* 25 Roux bottles containing broth-agar with the addition of 0·5 % Na lactate were sown with the organism and incubated for 24 hr., the culture then washed off and centrifuged, washed once in water and then made up into a thick cream (*c.* 35 ml.). This was poured into the mill and ground for 2 hr. by which time most of the cells had been disintegrated. The resulting juice was then centrifuged until microscopic examination showed it to be free from whole cells.

The juice was then tested for activity as aspartic deaminase and the results obtained are shown in Table V. 1 ml. each of the juice, $3M/250$ l-($-$)aspartate and $M/10$ phosphate buffer at pH 7·5 were shaken aerobically in a bath at 40°. The figures given are corrected for the blank of the juice. The activity was then redetermined in the presence of adenosine and also after dialysis with and without adenosine. It appears that neither dialysis nor the addition of adenosine has any appreciable effect on the activity. This may be due to the fact that the juice contains the optimal activating amount of adenosine already. Also, since

Table V. *Deamination of* 1-*aspartate by cell-free* coli-*juice*

	μg. NH_3-N liberated/ hr./ml. juice
(a) Juice + $M/250$ aspartate	97
(b) Juice + $M/250$ aspartate + 0·07 mg. adenosine	126
(c) Juice + 0·05 mg. adenosine	18
(b)–(c)	108
(d) Dialysed juice + $M/250$ aspartate	192
(e) Dialysed juice + $M/250$ aspartate + 0·07 mg. adenosine	107
(f) Dialysed juice + 0·07 mg. adenosine	18
(e)–(f)	89

Table VI. *Scheme of fractionation of* coli-*juice*

Cell-free *coli*-juice
|
½-sat. ammon. sulph.

Precipitate — Centrifugate

Dissolve in water — Saturate ammon. sulph.

½-sat. ammon. sulph. — Precipitate — Centrifugate

Precipitate — Centrifugate (discard) — Dissolve in water

FRACTION A — ½-sat. ammon. sulph.

Precipitate — Centrifugate

FRACTION B — Saturate ammon. sulph.

Precipitate — Centrifugate (discard)

FRACTION C
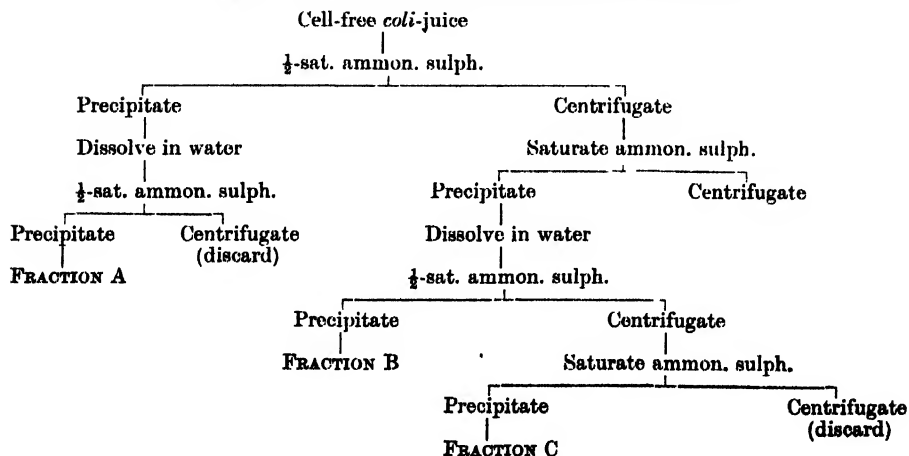
the bacteria had been grown in a manner different from that used for the organisms used in the first part of the work, it was possible that the juice contained other enzymes capable of deaminating aspartic acid.

Hence an attempt was made to fractionate the juice (Table VI).

Separation of aspartase I and aspartase II from cell-free coli-*juice.* The scheme provides three precipitates. Each of these was dissolved in water and then dialysed against distilled water until a blank of 10–20 μg. NH_3-N/ml. of preparation, was obtained. Each of the preparations was then tested for deaminase activity with l-(−)aspartic acid, the activity redetermined in the presence of adenosine at a final concentration of 1/10 mg./ml. and, finally, the preparation was incubated with toluene for 2 hr. and its activity then determined in the presence of adenosine (1/10 mg./ml.). The results of one such set of experiments is given in Table VII where the activities are expressed as μg. NH_3-N liberated per hr. per 100 mg. dry wt. of preparation. Fraction A has a small blank activity which is increased some 500 % by the presence of adenosine and its activity is completely abolished by incubation with toluene; fraction B appears to have no activity towards l-aspartic acid under any of the circumstances tested. Fraction C has an activity which is unaffected by added adenosine and is completely unaltered by toluene treatment. These experiments were carried out anaerobically. The course of deamination by fraction A under various conditions is set out more fully in Fig. 13.

Table VII. *Analysis of fractions from* coli-*juice*

	μg. NH_3-N/hr./100 mg. dry wt.		
	Fraction A	Fraction B	Fraction C
(*a*) *M*/250 aspartate	110	0	90
(*b*) *M*/250 aspartate + 0·1 mg. adenosine	650	0	75
(*c*) 0·1 mg. adenosine	70	0	0
(*b*)–(*c*)	580	0	75
(*d*) Fraction (after 2 hr. with toluene at 37°) + *M*/250 aspartate + 0·1 mg. adenosine	0	0	85
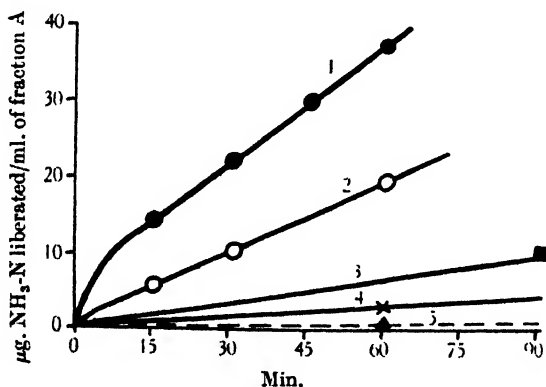
Fig. 13. Deamination of *M*/250 aspartate by fraction A: 1, + adenosine 0·1 mg. ml.; 2, + adenosine 0·03 mg./ml.; 3, no addition; 4, adenosine alone 0·1 mg./ml.; 5, same as 1, with toluene-treated fraction.

This fractionation leads to the separation of two distinct enzyme systems which deaminate l-(−)aspartic acid anaerobically. The enzyme of fraction C is unaffected by toluene or adenosine and must therefore be the one studied by

previous workers and called "aspartase" by Woolf [1929]. It is proposed that, in future, this enzyme should be called "aspartase I" and the enzyme present in fraction A whose activity depends upon the presence of adenosine and is completely inhibited by toluene treatment, "aspartase II". The enzyme separated from *Bact. fluoresc. liquefaciens* [Virtanen & Tarnanen, 1932] was probably, since toluene was used in the extraction, aspartase I.

Distribution of aspartase I and II in Bact. coli. It has been possible to demonstrate the presence of aspartase II in cultures of *Bact. coli* of both strains used in this laboratory and grown in the various conditions set out in Table VIII. Toluene treatment of the bacterial suspension completely inhibits aspartase II but, according to the work of Quastel & Woolf [1926], has no effect on aspartase I. Hence the Q_N determined after such treatment and measured during the initial stages of the deamination while the evolution of NH_3 is linear, gives a measure of aspartase I activity. From Fig. 11 we see that the rate of deamination is constant aerobically in the presence of a concentration of adenosine greater than the critical value and we can take this rate as a measure of the total activity. Assuming for the present that this total activity is the sum of the activities of aspartase I and II, we obtain that of aspartase II by difference. All the relevant figures are set out in Table VIII. The ratio of aspartase II/aspartase I shows that quantitatively the aspartase II is the more important enzyme in all cases. With strain I, aspartase I is negligible in the anaerobic cultures and it is with this strain that the greater part of the work has been carried out. Strain II shows approximately the same total activities for each type of culture as strain I but contains, in every case, a higher degree of activity due to aspartase I so that the ratios are of a lower order throughout.

Table VIII. *Analysis of deaminase activity and distribution of aspartase I and II in* Bact. coli

	Aerobic deamination Q_N		Toluene treated		Anaerobic deamination Q_N		Tol. treat.	Total activity	Aspartase I	Aspartase II	Ratio II/I
Adenosine	−	+	−	+	−	+	+				
Conditions of growth											
Strain 1:											
Anaerobic in broth	138	364	8	7	32	401	7	364	7	357	51
Broth in flask	150	250	23	6	32	114	4	250	4	246	62
Aerobic in broth	166	331	28	30	27	348	50	331	50	281	4
Aerobic on agar	96	121	6	6	17	127	9	124	9	115	13
2% glucose broth	34	75	12	15	26	83	20	75	20	55	3
Strain II:											
Anaerobic in broth	75	449	36	36	77	395	80	449	80	369	4·6
Aerobic in broth	312	437	70	48	114	400	60	437	60	377	6
2% glucose broth	54	95	32	32	40	106	32	95	32	63	2

It can now be seen why previous workers should have overlooked the existence of aspartase II. Woolf [1929] grew his cultures in broth in Roux bottles and so would have obtained the highest content of aspartase I. Growth was allowed to continue for 48 hr. by which time, as can be seen from Fig. 2, the total activity would have fallen to a low figure. The organisms were washed 3 times and the suspensions then aerated for 1 hr. so that a considerable "decay" of aspartase II activity would occur. The suspension was kept for use up to a fortnight and it is stated that the activity remained constant for 2 months whereas aspartase II

activity decays to zero within 12–24 hr. Finally, the actual experiments were carried out anaerobically when the small amounts of adenosine present would have no significant effect and also after toluene treatment which inhibits the action of aspartase II.

Effect of formate on deamination of l-aspartic acid by Bact. coli. In the early experiments of this series, a reactivation of a "decayed" suspension was obtained with boiled bacteria or with formate. The action of the boiled bacteria can now be explained since Mesrobianu [1936] has shown that *Bact. coli* contains an appreciable amount of adenosine and is a plentiful source of its precursors. The effect of formate remains to be investigated.

Figs. 14 and 15 show the effects on the anaerobic and aerobic deaminations of the addition of the critical amount of adenosine (1), $M/120$ formate (2), and of the two together (3). Aerobically, the formate has the same accelerating effect as adenosine but anaerobically, after a brief marked acceleration of the rate, the effect of formate is scarcely significant.
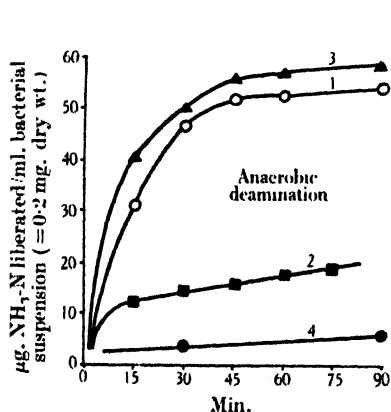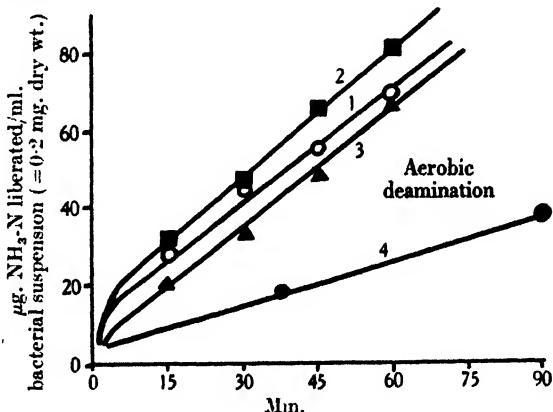
Fig. 14. Fig. 15.

Fig. 14. Anaerobic liberation of NH_3 from l-(−)aspartate by *Bact. coli* (I) in presence of: 1, adenosine 0·03 mg./ml.; 2, $M/120$ formate; 3, $M/120$ formate + adenosine 0·03 mg./ml.; 4, alone.

Fig. 15. Aerobic liberation of NH_3 from l-(−)aspartate by *Bact. coli* (I) in presence of: 1, adenosine 0·03 mg./ml.; 2, $M/120$ formate; 3, $M/120$ formate + adenosine 0·03 mg./ml.; 4, alone.

Fig. 16 shows the effect of increasing formate concentration on the aerobic Q_N. For comparison the effects of similar concentrations of adenosine are marked with a dotted line and it is seen that, to attain a maximum rate, a much higher concentration of formate than of adenosine is required but that the maximum rate reached is the same in both cases. With strain I, the critical concentration of formate would appear to be slightly greater than $M/1500$.

Fig. 17 shows the results obtained in an experiment with the aspartase II fraction of *coli*-juice in which the rate of deamination measured in μg. NH_3-N liberated per 30 min. per ml. of preparation is determined in the presence of various concentrations of adenosine (1). The deamination is followed aerobically and a maximum rate is obtained with a concentration of 0·125 mg./ml. adenosine. The experiment was then repeated in the presence of $M/6000$ formate (2) with the result that the maximum rate of deamination was reached in the presence of 0·0625 mg./ml. adenosine, so that the critical concentration of adenosine is halved by the presence of the formate. In the absence of adenosine, formate alone has no consistent or appreciable effect on deamination by the fraction.

Fig. 18 shows the results of an experiment similar to the last, carried out on a suspension of the bacteria. The critical concentration of adenosine for maximum rate of deamination was determined and gave the usual value of 1/30 mg./ml. The experiment was then repeated in the presence of $M/6000$ formate and again
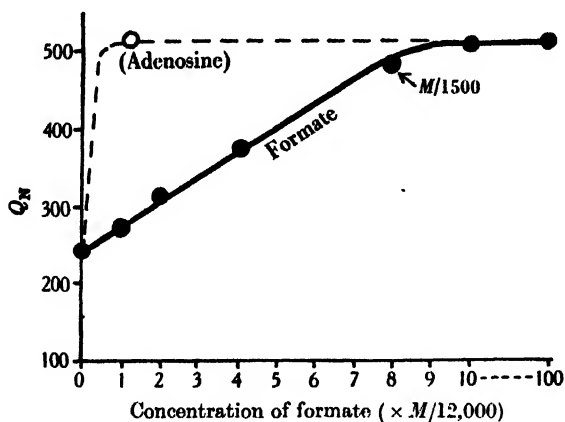
Fig. 16. Effect of formate (and adenosine) on aerobic deamination.
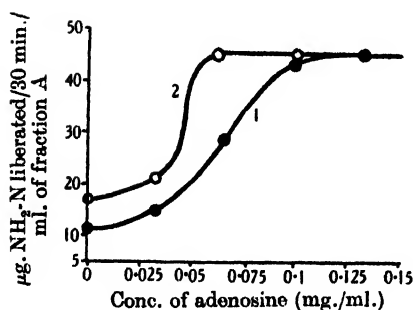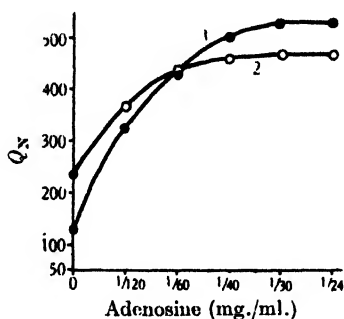
Fig. 17.   Fig. 18.

Fig. 17. Rate of deamination of l-(−)aspartate by fraction A of coli-juice in presence of: 1, varying concentrations of adenosine; 2, varying concentrations of adenosine + $M/6000$ formate.

Fig. 18. Rate of deamination of l-(−)aspartate by Bact. coli in presence of: 1, varying concentrations of adenosine; 2, varying concentrations of adenosine + $M/6000$ formate.

the optimal concentration of adenosine was approximately halved by this concentration of formate. Reference to Fig. 16 shows that $M/6000$ formate is much less than the critical concentration of formate required by strain I to give the maximum Q_N.

Since the presence of $M/6000$ formate halves the amount of adenosine that must be added to give a maximum Q_N, and $M/1500$ formate will give this maximum Q_N in the absence of added adenosine while the organism already contains a certain amount of adenosine, it is suggested that the presence of sufficient formate decreases the amount of adenosine required until the system is maximally activated by the natural adenosine of the organism.

Products of the action of aspartase II on l-(−)aspartic acid. A preparation of aspartase II was made according to the scheme set out above and the fraction dissolved in water and dialysed overnight. In this way 40 ml. of a solution of

aspartase II were obtained. This was set up in a large Thunberg tube with 25 ml. Na aspartate equiv. to 1 g. l-(−)aspartic acid; 50 mg. adenosine dissolved in 15 ml. water, and 20 ml. $M/5$ phosphate buffer at pH 7·5. The tube was evacuated and placed in a bath at 40°; 0·2 ml. of the contents was withdrawn every 30 min. and the NH_3-N determined to follow the course of the reaction. After 5 hr. when the deamination had almost stopped at *c.* 50 % completion the reaction was stopped by acidifying with H_2SO_4.

In a preliminary experiment, acid was added to a concentration of 10 % and the solution extracted with ether in a separating funnel. The ether extract was evaporated to dryness, the residue taken up in water and precipitated with conc. $AgNO_3$. The white precipitate obtained was filtered off, resuspended in water and decomposed with H_2S, the Ag_2S filtered off and the clear filtrate evaporated to small bulk. On standing about 20 mg. of colourless crystals were obtained. A portion of these, dissolved in water, gave a white precipitate with $AgNO_3$, decolorized $KMnO_4$ and Br water and gave the reactions typical of fumaric acid. M.P. (dec.) 200° in open tube with sublimation, M.P. 240° in sealed tube, which is considerably lower than the M.P. (287°) of pure fumaric acid. Thus the product was impure and represented a very low yield.

Accordingly the exp. was repeated. After 5 hr. incubation the reaction was stopped by acidifying slightly with H_2SO_4 and boiling to precipitate the protein which was then filtered off. The filtrate was neutralized and evaporated to 15 ml. and then extracted continuously with ether in a liquid extractor for 6 hr. The ether extract was then evaporated to dryness and the residue dissolved in 25 ml. of water.

The water solution was analysed for fumaric, succinic and malic acids according to the method of Needham [1927]. 1 ml. of the solution was neutralized with sat. $Ba(OH)_2$ to remove sulphate and phosphate; the precipitate was filtered off immediately and washed with water.

97 % alcohol was then added to a final concentration of 36 % and the pH adjusted to 7·0. 2 ml. conc. $AgNO_3$ were then added and the precipitate was filtered off on a Gooch crucible. After thorough washing with 60 % alcohol, the precipitate was redissolved in dil. H_2SO_4 and the total Ag estimated by titration in the cold against 0·01 N KCNS using iron alum as external indicator. The AgSCN was filtered off and the filtrate analysed for the above acids.

Malic acid was estimated by the method of Auerbach & Kruger [1923] and it was shown that, under the conditions of the experiment, fumaric acid could be estimated by titration at 40° in the presence of H_2SO_4 against 0·01 N KMnO₄ (1 ml. 0·1 N KMnO₄ = 1·27 mg. fumaric acid).

Table IX. *Analysis of residue from ether extraction*

Residue dissolved in 25 ml. water	
Total Ag obtained from 1 ml.	$= 11·8$ mg.
Fumaric acid/ml. estimated against $N/100$ KMnO₄	$= 2·47$ mg.$\equiv 4·7$ mg. Ag
Malic acid/ml. estimated polarimetrically	$= 4·1$ mg.$\equiv 6·6$ mg. Ag
Total silver/ml. due to known acids	$= 11·3$ mg.
Total malic acid in residue	$= 102·5$ mg.$= 102$ mg. aspartic acid
Total fumaric acid in residue	$= 61·8$ mg.$= 70$ mg. aspartic acid
Total aspartic acid equivalence of residue	$= 172$ mg.$\}$ 49 % recovery
Total aspartic acid disappeared (from NH_3 determinations)	$= 348$ mg.$\}$

From Table IX it can be seen that the total acids of the extract as determined by the Ag salts can be accounted for as malic and fumaric acids. However, the total yield of these acids will account for only 50 % of the aspartic acid that has

disappeared but Needham [1927] showed that malic acid cannot be extracted quantitatively from water solution owing to its unfavourable partition coefficient. Since it would appear that only malic and fumaric acids are produced, these can both be estimated accurately in the actual reaction mixture if this is first deproteinized and evaporated to a small bulk. Table X gives the results of such an experiment and it can be seen that the aspartic acid disappearing is satisfactorily accounted for by the malic and fumaric acids appearing.

Table X. *Products of aspartase II action*

Total aspartic acid disappeared = 285 mg.
Total malic acid formed   = 162·5 mg. ≡162 mg. aspartic acid
Total fumaric acid formed   =116·0 mg. ≡133 mg. aspartic acid
Equivalent total aspartic acid   = 295 mg.

Thus the action of the fraction A obtained from *coli*-juice upon *l*-(−)aspartic acid is to produce NH_3 together with fumaric and malic acids. In the extract, the sum of the fumaric and malic acids does not account for the aspartic acid disappearing, but if these are estimated in the reaction mixture itself, then complete equivalence is obtained.

The presence of fumarase in fraction A. The formation of both malic and fumaric acids in the last experiment suggests that the fraction A obtained from *coli*-juice contains fumarase in addition to aspartase II. This was shown to be the case by the following experiment.

40 ml. of a preparation of fraction A were incubated *in vacuo* with 20 ml. phosphate buffer at pH 7·5 and 500 mg. fumaric acid made up into 20 ml. of neutral solution. At intervals of ½, 1, 2, 4, 6 and 24 hr. a 5 ml. sample was withdrawn and added to 10 ml. of 14·2 % ammonium molybdate solution. A similar sample was added to 11 ml. of 6 % trichloroacetic acid. 1 ml. of glacial· acetic acid was then added to the first mixture and both samples were left in the dark for 3–4 hr. The protein was precipitated by both sets of reagents and was filtered off before the final estimation. The filtrate was made up in each case to 22 ml. for convenience and the malic acid estimated by the difference in rotation of the two samples [Auerbach & Kruger, 1923].

Table XI shows the formation of malic acid from fumaric acid in this experiment and thus demonstrates the presence of fumarase in the fraction.

Table XI. *Presence of fumarase in fraction A of coli-juice*

Initial concentration of fumaric acid = 6·25 mg./ml.
Increase in rotation (Hg green) in presence of molybdic acid due to 10 mg. malic acid under experimental conditions = + 0·80°.

Time	Increase in rotation in presence of molybdic acid	Malic acid mg./5 ml. sample	Malic acid mg./ml.
0	0	0	0
30 min.	+0·47°	5·25	1·05
1 hr.	+0·87	10·9	2·2
2 hr.	+1·35	16·9	3·4
4 hr.	+2·09	26·1	5·2
6 hr.	+2·11	26·4	5·3

SUMMARY

1. The deaminating activity of *Bact. coli* towards *l*-(−)aspartic acid varies (*a*) with the conditions of growth of the organism, and (*b*) with the age of the culture. The latter variation can be shown to be due to an alteration in the

chemical constitution of the growth medium consequent upon the metabolic activity of the cells.

2. Washed suspensions lose their activity upon standing and the rate of loss depends upon the dilution of the suspension.

3. The lost activity can be immediately recovered by the addition of boiled bacteria or formate to the suspension.

4. The recovery obtained with boiled bacteria resembles a coenzyme effect.

5. The coenzyme can be replaced by adenylic acid and its breakdown products, the most active being adenosine.

6. The addition of adenosine to the bacterial suspension results in a greatly increased rate of deamination aerobically or anaerobically.

7. A cell-free juice obtained by crushing and centrifuging *Bact. coli* will deaminate *l*-(−)aspartic acid.

8. A scheme for fractionation of the juice with $(NH_4)_2SO_4$ is given: this scheme results in the separation of two enzymes capable of deaminating *l*-(−)aspartic acid anaerobically. These enzymes are provisionally named:

Aspartase I which is unaffected by toluene and/or adenosine.

Aspartase II which is completely inhibited by toluene treatment, requires the presence of a coenzyme for its action, and is activated *in vitro* by adenosine.

9. The distribution of aspartase I and II in cultures of *Bact. coli* (two strains) grown under varying conditions has been determined.

10. Formate produces an activation of aspartase II aerobically but not significantly anaerobically.

11. Formate appears to reduce the amount of adenosine required to activate aspartase II.

12. The action of the aspartase II-fraction of *coli*-juice on *l*-(−)aspartic acid is to produce NH_3 and fumaric and malic acids.

13. The aspartase II-fraction of *coli*-juice also contains fumarase.

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CCVII. THE PRODUCTION OF INDOLE
BY SUSPENSIONS OF *BACT. COLI*

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WOODS [1935, 1] has studied the conversion of tryptophan into indole by washed suspensions of *Bact. coli*. He found that the process was an oxidation requiring 5 atoms O per mol. tryptophan. The conversion of tryptophan to indole was complete and the rate of disappearance of tryptophan was the same as the rate of formation of indole. The coli suspensions used by Woods were prepared from growths on trypsin-digested casein solidified with agar, and thus had always been produced in the presence of tryptophan. Happold & Hoyle [1935] also studied the oxidation of tryptophan and found on two out of three occasions that slight activity was present in suspensions grown in the supposed absence of tryptophan on Fraenkel's synthetic medium. Since this medium, however, contained natural asparagine [Dr Happold, private communication] it is possible that tryptophan might have been present as an impurity in biologically significant amounts. It was therefore decided to test the production of the enzyme under conditions in which tryptophan was certainly absent.

Technique

(1) *Medium used for growth of the suspensions.*

KH_2PO_4	...	...	4·5 g.
$(NH_4)_2SO_4$	...	...	0·5 g.
NH_4Cl ...	...	...	0·5 g.
$M/2$ Na lactate	...	50 ml.
N NaOH	...	...	26 ml.
Water to	...	...	950 ml.

This mixture was adjusted to pH 7·6, distributed in 9·5 ml. volumes in tubes and autoclaved.

The following solutions were prepared separately: $MgSO_4$, $7H_2O$, 0·4 % (autoclaved): ferrous ammonium sulphate $M/500$ in $N/50$ HCl (filtered); Na dithiodiacetate $M/200$ (filtered).

These were mixed in the proportions Mg 1·0, Fe 2·5, dithioacetate 2·0, and 0·5 ml. added per tube. The dithiodiacetic acid was prepared as stated by Fildes & Richardson [1937]. Na lactate was prepared from three times recrystallized Zn lactate.

The mixtures, having been prepared in the test tubes and inoculated were poured into 50 ml. Erlenmeyer flasks and incubated at 37° in air + 5 % CO_2.

All apparatus was treated in the usual way for excluding the presence of unknown substances.

(2) *The strains used.* Two laboratory cultures maintained on agar and four recent isolations were used. These gave the fermentation reactions of *Bact. coli*, *Bact. coli communis* and *Bact. acidi lactici*. All grew copiously after little hesitation on the medium described and in serial subculture from one drop of the previous culture in 10 ml. All produced indole rapidly when tryptophan

was added to the culture medium. Though some of the observations to be described were checked on more than one strain, a single strain was used as the standard. This was obtained from the cultures of the Bland-Sutton Institute and was described as No. 86 of the National Collection of Type Cultures. After 20–24 hr. growth the cultures were centrifuged and the supernatant liquid discarded. The bacteria were washed once with distilled water and suspended to a standard opacity in $M/18$ phosphate buffer (pH 7·6). The dry weight of bacteria from 1 ml. of this suspension was 1·5 mg.

(3) *Estimation of activity of the suspensions.* The bacterial suspension (7 ml.) was mixed in a 100 ml. Erlenmeyer flask with $M/18$ phosphate at pH 7·6 (12·5 ml.) and 0·5 ml. 0·175 % tryptophan, and incubated at 37°. The quantity of tryptophan used represented a possible maximum yield of indole of 25 μg./ml. Woods used four times this concentration.

The indole produced in the mixture was estimated by a simple method which appeared to be sufficiently accurate and gave results comparable with those of Woods.

A standard solution of indole was prepared containing 20 μg./ml. and from this a series of 10 dilutions with 25 % differences. One ml. of each dilution was mixed with 1 ml. of Ehrlich's indole reagent, prepared freshly from a sample of p-dimethylaminobenzaldehyde as colourless as possible. This reference series was remade after 4 hr.

The suspensions to be tested were centrifuged and the supernatant liquid diluted with water. One ml. of each dilution was mixed with 1 ml. Ehrlich reagent and after a few minutes tested against the reference series. The average of the estimates obtained from three or four dilutions was taken as the correct reading.

Various indole derivative tests by this technique at concentrations equivalent to 20 μg. indole/ml. gave negative results, namely indoleacrylic acid, indolepyruvic acid, indolepropionic acid, indoleacetic acid. Indolecarboxylic acid gave a mere trace of colour which was due to indole which the preparation contained.

The activity of suspensions of bacteria grown without tryptophan

Each of the six strains grown under the conditions stated actively converted tryptophan into indole. Fig. 1 shows examples of the action of different suspensions of one strain (No. 86, N.C.T.C.) grown on different occasions. No. 86 was equally active after 40 subcultures since its last contact with tryptophan.
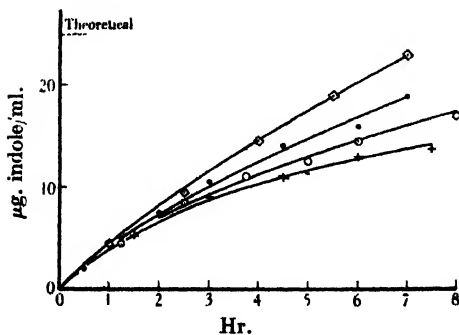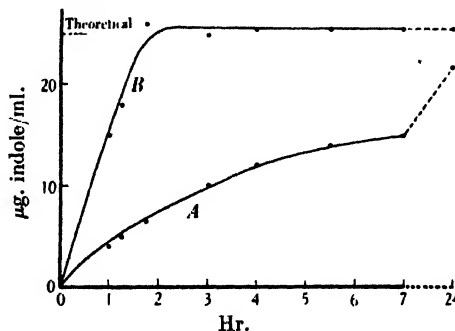
Fig. 1. Rate of production of indole by suspensions grown without tryptophan.

Fig. 2. Rate of production of indole by suspension A grown without tryptophan, compared with B grown with tryptophan.

The effect of growth in the presence of tryptophan

Parallel cultures were put up, one containing 0·5 ml. 0·175 % tryptophan per 10 ml. The suspensions were gathered after 22 hr. and gave the results shown in Fig. 2. The presence of the tryptophan did not increase the yield of the culture. Fig. 2 shows that growth in the presence of tryptophan much increased the activity of the suspension.

That the effect of growth in tryptophan is quantitative rather than qualitative is shown in Fig. 3. The suspension from a tryptophan culture was diluted serially with buffer until the initial activity was equal to that of the suspension grown without tryptophan. The course of the curves was exactly the same. In this experiment it was found that the activity of two suspensions could not be estimated from the initial courses of the curves. According to these it was assumed that the tryptophan culture was about five times more potent whereas it had to be diluted twenty-five times before its activity was the same as that of the culture grown without tryptophan.

These results obtained when tryptophan was present in the culture were in general confirmation of those of Woods and indicated that the less elaborate method used for estimating indole did not lead to any notable error.
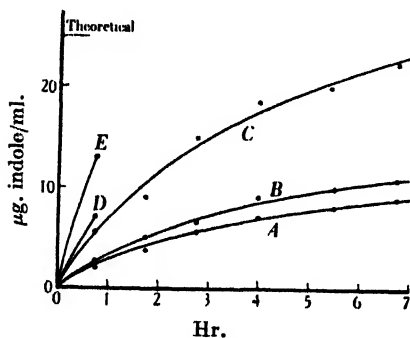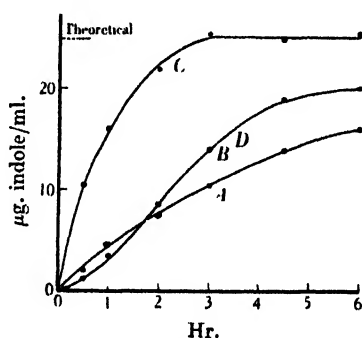
Fig. 3. Comparative activity of suspensions grown without tryptophan (A) and with tryptophan. B = tryptophan suspension diluted 1/25; C = 1/6; D = 1/4; E = undiluted.

Fig. 4. Effect of growth in glucose. A grown in lactate; B in glucose; C in lactate + tryptophan; D in glucose + tryptophan.

The effect of growth in glucose on the activity of the suspensions

It is a well-known phenomenon that the presence of glucose in a tryptophan-containing medium inhibits the production of indole by colon bacilli growing therein. Happold & Hoyle [1936] have concluded that this is due to a non-production of the "tryptophanase" enzyme in bacteria growing in the presence of glucose.

To test this possibility, flasks of the standard medium were prepared without, however, lactate; other ingredients were added as follows:

A, Medium + $M/2$ lactate 0·5 ml.
B, Medium + $M/2$ glucose 0·5 ml.
C, Medium + $M/2$ lactate 0·5 ml. + 0·175 % tryptophan 0·5 ml.
D, Medium + $M/2$ glucose 0·5 ml. + 0·175 % tryptophan 0·5 ml.

The cultures were grown for 20 hr. Those containing glucose were found to have given a smaller yield than those with lactate and thus the four suspensions

to be tested were made 1/3 the usual concentration. The centrifuged liquid of flasks *B* and *D* still contained glucose. Flask *C* contained quantities of indole equiv. 100 % conversion of tryptophan. Flask *D* contained traces of indole, < 1 μg./ml. and approximately 100 % of unconverted tryptophan. The presence of glucose in the culture had therefore almost entirely inhibited the production of indole. Fig. 4 shows the action of the washed suspensions on tryptophan. It will be noticed that the suspension grown in glucose, after a preliminary delay which appears from other experiments to be constant, showed at least as much activity as that grown in lactate and that the presence of glucose had entirely inhibited the excess activity due to the tryptophan.

Thus it is possible to distinguish two "fractions" of activity in suspensions grown in the absence and presence of tryptophan.

(*a*) One fraction which is always present in *Bact. coli*, which does not depend for its production upon the presence of tryptophan in the external medium and which is unaffected by glucose therein. This fraction may be looked upon as "constitutive" in the sense of Karström [1938].

(*b*) Another fraction which is produced as a result of tryptophan in the external medium, which action is inhibited by the presence of glucose therein. This fraction may be called "adaptive".

It may readily be shown colorimetrically that the washed bacteria contain tryptophan which they have synthesized from NH_3 and thus it is possible that both fractions of activity result from an action of tryptophan.

The effect of indole upon the activity of suspensions

Three suspensions were made from cultures grown without added tryptophan. To each was added tryptophan to make a final conc. equivalent to 25 μg. indole/ml. To one was also added ½ equiv. of indole and to another 1 equiv. Fig. 5 shows that the activity of the suspension was entirely inhibited by 1 equiv. indole and largely inhibited by ½ equiv.
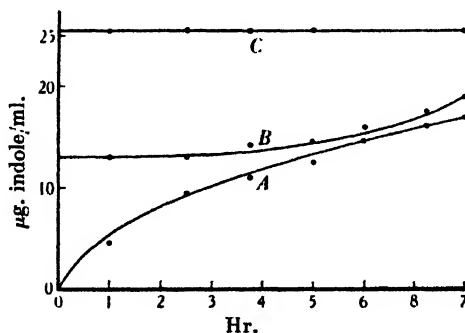
Fig. 5. Inhibition by indole. *A* = action of suspension on tryptophan; *B* on tryptophan + ½ equivalent of indole; *C* on tryptophan + 1 equivalent of indole.

The same result was obtained when suspensions were used from cultures grown in tryptophan, but only when they were diluted to an activity similar to that of a suspension grown without tryptophan. When these suspensions were less diluted, more indole was required to produce a total inhibition. Thus the inhibitory action of indole depends upon the extent of enzyme surfaces present rather than upon the concentration of the substrate. The suspensions which had been proved to be inactive in the presence of tryptophan + indole

were shown to be potentially active by washing and resuspension in tryptophan. None of the indole derivatives already mentioned had any effect on the activity of suspensions under these conditions.

The effect of growth in the presence of indole and derivatives

In view of the effect of tryptophan in increasing the activity of suspensions of bacteria grown in a medium containing this substance, the actions of indole itself and of derivatives were tested.

As has been found by others the presence of indole in a culture is inhibitory to growth. In the present series when indole was added in equivalent concentration to the tryptophan, growth was delayed until the 2nd day and was not normally heavy until the 3rd. With indoleacrylic acid very little growth took place during the first 3 or 4 days but a heavy growth was found on the 4th or 5th. No other indole derivative was found to inhibit. By means of serial subculture in the inhibitory solutions, it was possible to banish the inhibition by indole and to reduce that by indoleacrylic acid so that full growth took place on the 2nd day. In the following experiment therefore the activities of suspensions were tested after 24 hr. growth except in the case of indoleacrylic acid, in which the bacteria were grown for 48 hr. The serial subcultures did not affect the general result of other similar experiments, but allowed a more exact comparison of activity to be made.

All cultures were prepared as before. Indole and its derivatives were dissolved in water or when necessary in $M/20$ NaOH in conc. equiv. 0·175 % tryptophan and 0·5 ml. portions of these solutions were added to 10 ml. of medium, after sterilization by Seitz filtration.

The following derivatives were used: skatole, indolecarboxylic acid, indolepyruvic acid, indolepropionic acid, indoleacetic acid, and indoleacrylic acid.

The suspensions were washed as usual and put up with tryptophan equiv. 25 μg. indole/ml.

Fig. 6 shows that an equal stimulation of the activity of the suspensions had resulted from growth in the presence of indole and indoleacrylic acid though the other compounds had no stimulatory effect.

Fig. 6. Activity of suspensions grown in (A) tryptophan; (B) indole or indoleacrylic acid; (C) indole-pyruvic, -propionic, -carboxylic or -acetic acids, or in skatole, or with no addition.

The production of indole from indoleacrylic acid

It has been shown by Woods [1935, 2], Happold & Hoyle [1935] and others that suspensions of *Bact. coli* are unable to produce indole from indoleacrylic acid or any of the other derivatives mentioned. These results have been confirmed in the present work.

On the other hand in experiments in which the bacteria were grown in the presence of these substances, the result was different. Indole was formed from indoleacrylic acid added to the culture but not from the other derivatives even after 5 days' incubation.

The production of indole from indoleacrylic acid appears to have little relation to the amount of growth which has taken place. For instance in two experiments in which the growth was moderate in 24 hr. as a result of continuous subculture in indoleacrylic acid, the production of indole was only 1 μg./ml.

and < 1 µg./ml. (theoretical 50 µg./ml.). On the 2nd day when growth was full, 2·5 and 4 µg./ml. had been produced. In another experiment using a strain unadapted to indoleacrylic acid the relation of growth to indole was as follows:

Days	Growth	Indole µg./ml.
1st	0	0·0
2nd	Trace	8·5
3rd	Trace	23·5
4th	+ + +	33·0

Under similar conditions the production of indole by suspensions grown in tryptophan would have been complete (50 µg./ml.) in less than 24 hr.

The mechanism by which the indole is produced is not clear but as a suspension of *Bact. coli* is unable to oxidize this substance directly, it would appear probable that under the conditions of growth *Bact. coli* is able slowly to synthesize tryptophan from indoleacrylic acid and that this is then oxidized to indole.

These results allow the conclusion that, whatever significance may be attached to the stimulation of activity by growth in tryptophan or indole, a similar significance need not be associated with the action of indoleacrylic acid, since this could be ascribed to the indole produced.

All workers are agreed that there is no experimental evidence to suggest that the oxidation of tryptophan to indole involves a passage through any of the theoretically possible intermediates tested. The present results with indoleacrylic acid are of interest in connexion with Raistrick's suggestion [quoted Cole, 1933] that this substance produced by a deamination of tryptophan may be the first step in the production of indole. If, as seems probable, *Bact. coli* can synthesize tryptophan from indoleacrylic acid the reverse process should also be possible, in agreement with Raistrick's idea. In practice it is not possible to demonstrate this action but this may merely be due to the much greater rapidity with which tryptophan is oxidized by another route. In any case the evidence still remains that indoleacrylic acid cannot be oxidized further and therefore this potential deamination of tryptophan can hardly be a step in the production of indole.

It appears to be generally held that the formation of indole from tryptophan probably takes place in steps catalysed by a series of enzymes. On the other hand if the detachment of the indole ring took place in one step under the influence of one enzyme it would be more easy to account for the phenomena noted here. The fact that growth in tryptophan or in indole leads to an increase in the enzyme activity is more in accordance with a view that these are substrate and end-product of one enzyme, rather than substrate and end-product of two different enzymes. Further the fact that indole completely inhibits the action on tryptophan is also reminiscent of the well-known inhibition of enzymes by their own end-products.

SUMMARY

1. Suspensions of *Bact. coli* grown in the absence of tryptophan are always capable of oxidizing tryptophan to indole.

2. When grown in the presence of tryptophan, the activity is some twenty-five times greater.

3. The activity of the former suspensions is unaffected by the presence of glucose in the medium; the excess activity of the latter is entirely inhibited by glucose.

4. The activity of suspensions is inhibited by indole.

5. The activity of suspensions is increased by growth in the presence of indole or indoleacrylic acid. Other indole derivatives are inactive in this respect.

6. Indole is produced during growth in the presence of indoleacrylic acid, but not in the presence of other derivatives.

7. It is suggested that a growing culture can synthesize tryptophan from indoleacrylic acid.

I am much indebted to Mr D. D. Woods for helpful criticism and advice in the presentation of these results. I have also to thank Mr Woods and Dr F. C. Happold for the supply of some of the indole derivatives used.

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CCVIII. PHYSICAL PROPERTIES OF BUSHY STUNT VIRUS PROTEIN

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It has not hitherto been possible to determine accurately the molecular size or weight of any of the virus proteins. X-ray diffraction patterns show in the case of tobacco mosaic virus protein that the cross sectional area of the rod-shaped molecules is 20,100 sq. Å. [Bawden *et al.* 1936] and assuming the rod to be cylindrical this corresponds to a diameter of approximately $16\,m\mu$. The same technique fails to give any indication of the length of the molecule and it is not at all certain that the molecular units have all the same length. Sedimentation, osmosis and diffusion measurements are also unsatisfactory when applied to the anisotropic plant viruses owing to the combination of large molecular size with gross asymmetry which they possess.

Some attempts have recently been made to apply to solutions of the anisotropic virus proteins equations relating the viscosity of a suspension of rods to the dimensions of the rods themselves. Frampton & Neurath [1938] and Lauffer [1938] deduced from viscosity measurements that the length of the tobacco mosaic molecule is 35–40 times the width. For a width of $16\,m\mu$ the length is therefore 560–$640\,m\mu$ and the volume of the molecule can be calculated. If the density is $1\cdot37$ [Bawden & Pirie, 1937], the molecular weight by this procedure is 94–107×10^6, or approximately 100 millions. An alternative method of obtaining the molecular weight is to calculate the dissymmetry constant from the ratio of molecular length to width and to substitute this in the sedimentation equation. This method gives a weight of 42 millions [Lauffer, 1938]. Assuming, on the other hand, that the dissymmetry constant is $1\cdot3$, a value shared by a number of non-spherical proteins, a molecular weight of only 17 millions is obtained [Svedberg & Eriksson-Quensel, 1936]. The value of $1\cdot3$ is the lowest which could reasonably be proposed for a substance with the anisotropic properties of tobacco mosaic protein. Thus it seems that the molecular weight of the tobacco mosaic protein is more than 17 millions—probably considerably more—but less than 100 millions.

Recently a fully crystalline virus protein has been isolated [Bawden & Pirie, 1938, 1], the molecule of which appears to be spherical. This protein is obtained from the tomato plant infected with Bushy Stunt disease and is capable of transmitting the infection. It shows no anisotropy of flow and crystallizes in forms belonging to the cubic system. These properties suggest that the molecule is symmetrical and therefore adapted to exact measurements of size and weight by familiar physical methods. Our main object in the work described here has been to determine the size and weight of the virus protein molecule, but the opportunity has also been taken of investigating certain other properties, notably the electrophoretic behaviour.

The specimens of Bushy Stunt virus protein used in this work were prepared for us by Bawden & Pirie and a full description of the preparation is to

be found in their paper [1938, 2]. One specimen had been electrodialysed until the ash value, measured as sulphate, was 4 %. The solutions were colourless and water-clear by transmitted light.

Partial specific volume. This was determined pyknometrically at 25° and the values 0·743 and 0·734 obtained. The mean of 0·739 is approximately the same as Bawden & Pirie's value for tobacco mosaic virus protein and falls within the density range of the proteins as a class.

Sedimentation constant. Table I contains data from a series of sedimentation velocity determinations. These were carried out in the Svedberg equilibrium centrifuge at 21° and at a speed of 18,120 r.p.m. corresponding to a force of 20,000 times gravity in the middle of the fluid column. The ultraviolet light absorption technique was used for optical observation and the source of light was a high pressure mercury vapour lamp. A band in the ultraviolet was selected by means of chlorine and bromine gas filters. Exposure times of 15–20 sec. were used with Ilford Ordinary plates.
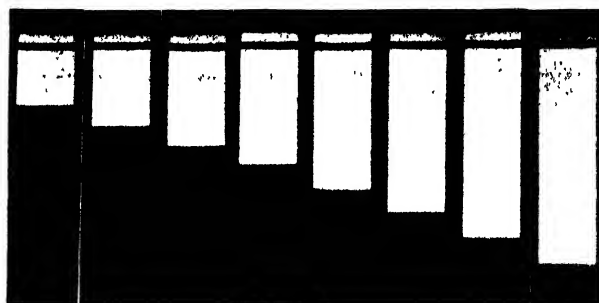
Fig. 1. A series of sedimentation pictures of the Bushy Stunt virus protein, 0·27 % in 0·02 M acetate buffer. Centrifugal force 20,000 g. 9 min. between each exposure.

Table 1. *Sedimentation velocity of Bushy Stunt virus protein*

Protein concentration	Buffer composition	pH	$S_{W, 20} \times 10^{13}$
0·11	0·01 M CH$_3$COONa; 0·01 M CH$_3$COOH	4·70	147
0·20	0·1 M citric acid; 0·018 M NaOH	2·40	138
0·20	0·0013 M KH$_2$PO$_4$; 0·0029 M Na$_2$HPO$_4$; 0·1 M NaCl	7·10	158
0·24	0·017 M CH$_3$COONa; 0·003 M CH$_3$COOH	5·46	153
0·25	0·003 M CH$_3$COONa; 0·017 M CH$_3$COOH	3·99	151
0·25	0·004 M CH$_3$COONa; 0·016 M CH$_3$COOH	4·12	142
0·25	0·05 M H$_3$BO$_3$; 0·014 M NaOH	8·70	141
0·26	0·006 M CH$_3$COONa; 0·014 M CH$_3$COOH	4·32	141
0·27	0·0005 M CH$_3$COONa; 0·0195 M CH$_3$COOH	3·40	143
0·29*	0·0013 M KH$_2$PO$_4$; 0·0029 M Na$_2$HPO$_4$; 0·1 M NaCl	7·10	145
0·34	0·01 M CH$_3$COONa; 0·01 M CH$_3$COOH	4·70	150

Mean $S_{W, 20} = 146 \times 10^{-13}$

* Electrodialysed prior to addition of buffer.

Fig. 1 shows a series of absorption pictures taken from one of the experiments in Table I, and typical of them all. The boundary is that of a perfectly homogeneous protein and shows a degree of sharpness which hitherto has only been obtained with some of the heavier haemocyanins [cf. Eriksson-Quensel & Svedberg, 1936, 1, 2]. One preparation which had been electrodialysed prior to the

addition of buffer showed the same perfect boundary sharpness as the others. The mean value of the sedimentation constant is 146×10^{-13}. The tobacco mosaic and cucumber virus proteins all give sedimentation constants higher than this, viz. $170–240 \times 10^{-13}$ [cf. Svedberg & Eriksson-Quensel, 1936; Wyckoff et al. 1937; Price & Wyckoff, 1938] but two plant viruses have smaller values, viz. latent potato mosaic (or "X" virus) for which $S_{W, 20} = 113$ [Loring & Wyckoff, 1937] and tobacco ring spot virus for which $S_{W, 20} = 115$ [Stanley & Wyckoff, 1937].

pH stability. From Table I it is seen that inside the range pH 2·4–8·7 the protein is homogeneous and unchanged in molecular size. This appears to be true for long periods at the hydrogen ion concentrations stated. After 1 hr. at pH 10 the protein consists of two components with sedimentation constants of 112 and 136×10^{-13}. Precisely the same splitting into components of 112 and 136 is found $\frac{1}{2}$ hr. after adjusting the pH to 1·3. After 48 hr. at this pH however only one boundary can be seen. This boundary is relatively sharp and sediments at a rate corresponding to $S_{W, 20} = 66 \times 10^{-13}$.

The pH stability range is thus a comparatively wide one, viz. pH 2·40–8·70. It is of interest that nowhere inside this range are any signs to be found of a minimum solubility zone in which molecular aggregation occurs such as is shown by some of the anisotropic virus proteins [cf. Bawden & Pirie, 1937].

Sedimentation equilibrium. Three determinations have been made at different protein concentrations. The height of fluid column used was approximately 2 mm. and the equilibrium centrifuge was run at the lowest speed available, viz. 1500 r.p.m., corresponding to a force of 150 times gravity at the centre of the fluid column. The scale method based on Lamm's refraction studies [Lamm,
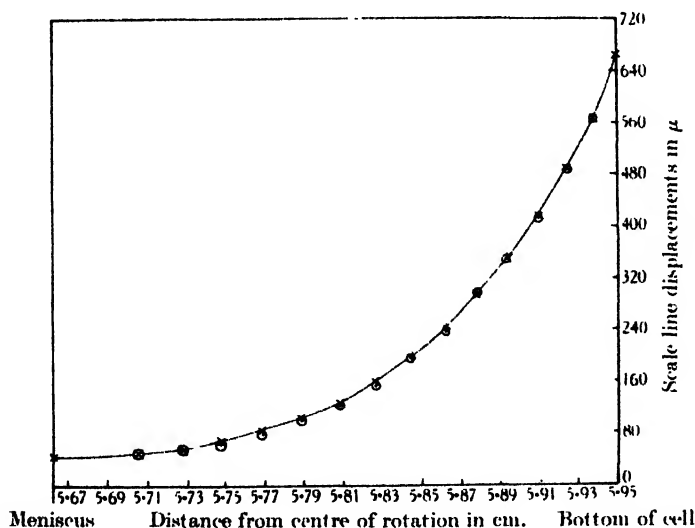
Fig. 2. ⊙ After 48 hours. × After 96 hours.

1937] was used, and the scale photographs were taken with light of wave-length $366 \, m\mu$. The superior accuracy of this method is especially apparent with the very steep equilibrium concentration gradients given by the heavier proteins. The temperature throughout was 20° and it was found that equilibrium was almost attained in 48 hr. and was complete after 96 hr. Fig. 2 shows the curves

obtained after 48 and 96 hr. in the same experiment. To obtain the concentration curve from this x, dn/dx curve a procedure recently described by Pedersen [1937] was used with advantage. The fluid column is divided into layers 0·02 mm. in thickness and the concentration in the middle layer is given a hypothetical value (C_0), for example the value of the protein concentration at the beginning of the experiment. By means of the concentration (or refraction) increments obtainable from the x, dn/dx curve the hypothetical mean concentration of an adjacent layer C_1, equal to $C_0 + dc/dx \times 0.02$, is obtained and by repeating this procedure a series of mean concentrations corresponding to all the layers from the meniscus to the bottom of the cell is obtained. This enables a concentration curve to be constructed and the integral of this curve from the meniscus to the bottom of the cell should be equal to the total amount of protein in the cell. In order that this may be so a factor equal to the difference between the curve integral and the known amount of protein in the cell divided by the height of fluid column is then added (or subtracted) from the individual hypothetical layer concentrations, and a true concentration curve constructed. From the concentration values in each adjacent pair of fluid layers the molecular weight is calculated from the formula [Svedberg, 1926]

$$M_W = \frac{2RT \ln (C_2/C_1)}{(1 - Vp)\, \omega^2 (x_2^2 - x_1^2)},$$

in which M_W is the molecular weight average and the other symbols have their customary significance.

The molecular weight may be determined by an alternative formula [cf. Pedersen, 1937], viz.

$$M_Z = \frac{2 RT \log_n \left(\frac{Z_2 x_1}{Z_1 x_2}\right)}{(1 - Vp)\, \omega^2 (x_2^2 - x_1^2)},$$

in which the Z values are scale line displacements in arbitrary units.

Table II. *Results of a typical sedimentation equilibrium experiment. Concentration of protein at start 0·335°/$_o$ in 0·02M. Acetate buffer pH 5·17. Cell 4 mm. thick. Speed 1530 r.p.m. Bottom of cell 5·95 cm. from axis of rotation $t = 20°$*

| | | | | Molecular weight × 10⁶ | |
| | | | | Weight-average (M_W) | Z-average (M_Z) |
x	z in μ	dc/dx	C %		
5·95	662	11·1	1·054		
5·93	515	8·64	0·962	7·22	7·55
5·91	412	6·91	0·807		
5·89	329	5·52	0·682	7·36	6·80
5·87	264	4·43	0·583		
5·85	211	3·55	0·503	7·70	6·72
5·83	168	2·83	0·440		
5·81	133	2·23	0·389	8·26	7·27
				Mean 7·64	7·09

Table II shows the results of a typical experiment and it will be seen that the variation in M_W or M_Z values with height in the cell is comparatively small. This is a reliable criterion that equilibrium has been established and that we are concerned with a homogeneous protein.

In Table III are shown the mean M_W and M_Z values for three equilibrations. The mean molecular weight value of all the determinations is 7,600,000.

Table III. *Results of three sedimentation equilibrium determinations on Bushy Stunt virus protein*

Protein concen-tration	Buffer composition	pH	Mean molecular wt. $\times 10^6$	
			M_W	M_Z
0·335	0·015 M CH$_3$COONa; 0·005 M CH$_3$COOH	5·17	7·64	7·09
0·220	0·015 M CH$_3$COONa; 0·005 M CH$_3$COOH	5·17	8·15	7·22
0·270	0·001 M CH$_3$COONa; 0·039 M CH$_3$COOH	3·40	8·01	7·42
			Mean 7·93	7·24

Size and weight of the molecule. The sedimentation constant is defined as the molecular velocity under unit force in water at $20°$. Since the force of gravity on a spherical particle is $\frac{4}{3}\pi r^3 (\rho_v - \rho_s)$, the force required to produce unit velocity is

$$\frac{4\pi r^3 (\rho_v - \rho_s)}{3 \times S_{W, 20}},$$

where ρ_v is the density of the protein and ρ_s is the density of the solvent.

According to Stokes' law this force is equal to $6\pi\eta r$, where η is the viscosity of the medium.

$$6\pi\eta r = \frac{4\pi r^3 (\rho_v - \rho_s)}{3 S_{W, 20}},$$

$$r^2 = \frac{9\eta S_{W, 20}}{2 (\rho_1 - \rho_s)}.$$

Substituting $S_{W, 20} = 146 \times 10^{-13}$ and $\rho_1 = 1·353$ for Bushy Stunt protein we find the radius of the molecule to be $13·7 m\mu$ and the weight 8,800,000.

The agreement between the molecular weight obtained from sedimentation equilibrium measurements and from sedimentation velocity measurements assuming Stokes' law indicates that the molecule does not depart markedly if at all from a symmetrical shape.

Electrophoretic mobility. Measurements of the electrophoretic mobility of the virus protein were made with the Tiselius apparatus [Tiselius, 1937], the migration of the protein boundary being followed photographically by the "Schlieren" method (Fig. 3). A sodium vapour lamp was used as a light source and exposures made on Ilford Panchromatic plates.

The mobility was measured at $0°$ in acetate buffers, total acetate $0·02 M$, the protein concentration being maintained between the limits $0·25–0·35$ g./100 ml. Corrections were applied for the viscosity effect of the buffer salts, and for the hydrostatic displacement [Tiselius, 1930]. Both of these corrections amounted to less than 1%.

The material showed the behaviour characteristic of a homogeneous substance, a single sharp Schlieren band being maintained throughout the experiments over the range of pH studied.

Details of the mobility experiments are given in Table IV and Fig. 4.

Table IV. *Electrophoretic mobility of Bushy Stunt virus protein*

pH	Mobility (u) cm.$^{-2}$ V.$^{-1}$ sec.$^{-1} \times 10^{-5}$	Direction
3·80	1·79	Cathodic
3·99	0·73	,,
4·12	0·15	Anodic
4·32	0·58	,,
4·70	1·73	,,
5·17	3·46	,,
5·47	4·59	,,

The isoelectric point of the virus protein was found to be $pH_0 = 4.11$ and the slope of the mobility curve at the isoelectric point, $du/d\,(pH_0) = 5.0 \times 10^{-5}$.
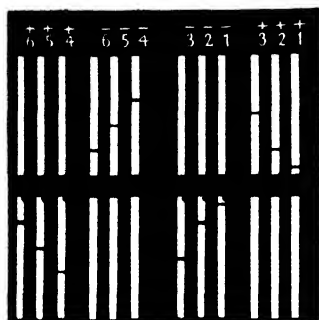
Fig. 3.
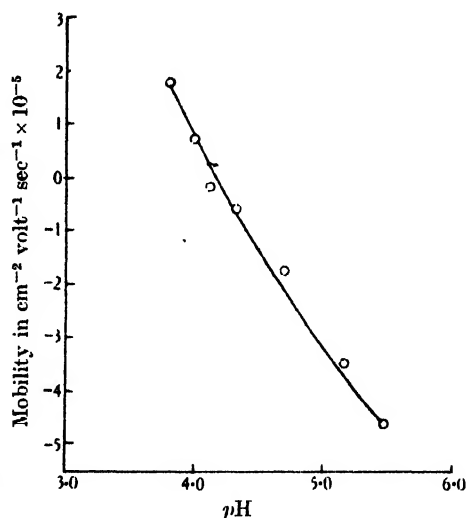
Fig. 4.

Fig. 3. Electrophoretic migration of Bushy Stunt virus protein in $0.02\,M$. Acetate pH 6.2. Potential gradient 9.5 V. cm. A single sharp boundary is present in all the exposures.

Fig. 4. Mobility-pH curve for Bushy Stunt virus protein in $0.02\,M$ acetate buffer.

Specific refractive increment and optical dispersion. The refractive increment of a solution of the protein in $0.01\,M$ NaCl at the isoelectric point was measured for three wave-lengths in the mercury spectrum using a photographic technique [cf. *Handbuch der Physik*, **18**, 668, 1927]. The protein concentration was determined by drying to constant weight *in vacuo* at room temperature. The results are given in Table V.

Table V. *Specific refraction increment of Bushy Stunt virus protein at different wave-lengths.* $T = 23°$.

Wave-length mμ	Specific refraction increment
366	0.00178
436	0.00170
546	0.00164

SUMMARY

The partial specific volume, sedimentation constant, molecular weight, electrophoretic mobility, isoelectric point and specific refraction increment of the crystalline nucleoprotein causing Bushy Stunt disease in the tomato plant have been determined. The protein is exceptionally homogeneous both with respect to size and charge and no evidence of an asymmetrical molecular shape could be found.

One of us (R. A. K.) is in receipt of a grant from the Medical Research Council.

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CCIX. THE MANOMETRIC DETERMINATION OF AMINO-ACIDS

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In a recent number of this *Journal* Mason [1938] has published under the above title a method depending upon the measurement, in the Van Slyke-Neill [1924] apparatus, of the CO_2 evolved from the carboxyl groups of amino-acids boiled with ninhydrin. This method had already been published by Van Slyke & Dillon [1936], in a paper which Mason quotes only as having "suggested" the method "as feasible". Although Van Slyke & Dillon's paper was presented as a preliminary report, the method was described in sufficient detail for exact repetition, and the nature of the results with different types of amino-acids, with polypeptides, and with other types of organic acids was stated. A supplementary description, with some improvements, was later published by Van Slyke & Dillon [1938] in Sørensen's Jubilee Volume, 3 months before the appearance of Mason's report. The method as republished by Mason [1938] differs in no significant detail from that originally outlined by Van Slyke & Dillon [1936].

Because it depends on the presence of both COOH and NH_2 or C—NH—C groups in neighbouring positions, the method appears to be the most specific yet devised for measurement of amino-acids in biological mixtures. For example, D. A. MacFadyen finds in the writer's laboratory that it can be applied directly to serum without removal of proteins. The full report on the method, including the results of Dillon and MacFadyen on which it and its applications are based, will appear in the near future.

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CCX. PHENAZINE COMPOUNDS AS CARRIERS IN THE HEXOSEMONOPHOSPHATE SYSTEM

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CERTAIN phenazine compounds resemble the flavins in that they can readily form semiquinones, to which property, generally very rare in dyestuffs, Michaelis *et al.* [1936, 1, 2] attach much importance in determining the catalytic activities of the flavin enzyme and vitamin B_2.

Phenazine methiodide [Dickens, 1936, 1] like pyocyanine [Friedheim, 1934] and certain flavin compounds [Laser, 1934] has an action on tissue metabolism which is quite different from that of most of the usual oxidation-reduction dyestuffs [Dickens, 1936, 1]. In tissues exhibiting strong aerobic glycolysis, particularly tumour tissue, the respiration is increased and the aerobic glycolysis falls in the presence of these compounds which thus appear to exert a control of the Pasteur reaction [cf. Dickens, 1936, 2]. While the action of the simpler phenazines is rapid, enabling its detection in short experiments by the tissue-slice method, that of the flavins can only be demonstrated in tissue culture experiments, presumably owing to slow absorption or slow transformation into the active carrier. On the other hand the easily diffusible phenazines cause some inhibition of the anaerobic glycolysis also [Dickens, 1936, 1], so that this is not a simple augmentation of the Pasteur effect. This group of substances produces such unusual effects that an attempt has been made to elucidate the mechanism of their action by a study of the catalytic role of the phenazines in an isolated enzyme system. Weil-Malherbe [1937, 2] has studied the action of phenazine methochloride and pyocyanine in the α-hydroxyglutaric system.

In the experiments described below we have prepared some new phenazine compounds and used them as substitutes for the flavin enzyme of Warburg in the hexosemonophosphate system, comparing their catalytic activities with those of other carriers. We have also measured the oxidation-reduction potentials of a number of phenazines for correlating our results. The experiments reveal new aspects of the common features of the phenazines and flavin compounds, and it is shown that in the hexosemonophosphate system the catalytic activity of some of these compounds much surpasses that of the ordinary oxidation-reduction dyestuffs studied.

Green *et al.* [1934] used pyocyanine as carrier in this system in their studies on carrier-linked reactions. Later Ogston & Green [1935] stated their objections to the description of the flavin-phosphate-protein complex as an enzyme. In the following we have retained the term flavin enzyme, since the question cannot be regarded as settled.

Enzyme and substrate preparations

Enzyme preparations. Many of the experiments were made on preparations of "Zwischenferment" [Negelein & Gerischer, 1936] and coenzyme II of purity 1, generously supplied by Prof. Warburg, who also gave us a sample of flavin

enzyme purified by the $CHCl_3$ method [Warburg & Christian, 1933] containing in the 10% solution $4 \cdot 1 \times 10^{-8}$ g.mol. pigment per ml. as estimated by Prof. Warburg. For use, 0·1 mg. dry Zwischenferment per vessel was dissolved in 2 ml. $M/100$ phosphate buffer. For other experiments we used a simple enzyme preparation made from Lebedew fluid prepared from Löwenbräu, Munich, bottom beer yeast as follows. The finely ground yeast, air-dried, was incubated for $2\frac{1}{2}$ hr. with 3 vol. water at 35°. After centrifuging, the clear fluid was diluted with 5 vol. distilled water and $0 \cdot 15 N$ acetic acid was added with stirring and ice cooling, to give pH 4·6 (the volume of acetic acid required is about half the vol. of Lebedew fluid taken). The white precipitate was collected at the centrifuge, washed with $0 \cdot 02 M$ acetate buffer pH 4·6, and dried quickly *in vacuo* over P_2O_5. The yield was about 2·5 g. per 250 g. dried yeast. If necessary, the enzyme may be dissolved in $0 \cdot 02 N$ ice cold NaOH and, after centrifuging from some insoluble residue, reprecipitated by acetic acid as before. It may be kept in the refrigerator for several weeks in the dry state without loss of activity. For use it was dissolved (10–20 mg. per vessel) in ice cold $N/100$ NaOH or $M/100$ phosphate buffer with addition of NaOH to give a final solution of the required pH, and centrifuged. Although this enzyme is much cruder than the Warburg material, with the quantity taken the blank was zero or very small and the rates of O_2 uptake were equal with the two preparations.

Coenzyme II of purity 0·2 was prepared from horse blood corpuscles by the method of Warburg & Christian [1936], "1 and 2 Schritte". Of this 0·1 mg. per vessel used instead of 0·02 mg. of the preparation of purity 1 gave the same rate of O_2 uptake.

Substrate. Ba hexosemonophosphate was prepared from the action of dialysed muscle extract on glycogen [Ostern *et al.* 1936]. It was found essential to allow full digestion to reduce the glycogen content to a minimum, but traces nevertheless remained. Prof. Robison very kindly analysed a specimen as follows. Found: total P 7·4%; inorg. P 0·1%; H. and J. reduction 32·9; I.V. 32·3; Seliwanoff 5·6; $[\alpha]_{5461}$ 23·4°; glycogen +. Calc. for $C_6H_{11}O_9PBa$: P 7·85%. The analysis is that of a mixed hexosemonophosphate [cf. Robison, 1932] containing a little glycogen.

For the preparation of the solution of the Na salt, 0·145 g. Ba salt was ground with 0·354 g. Na_2SO_4, $10H_2O$ and diluted to 5 or 10 ml. after the addition of 0·125 ml. N HCl. The centrifuged solutions contained respectively 0·18 or 0·09 M Na hexosemonophosphate (P analysis).

Phenazine compounds

Methylphenazonium salts. Phenazine methosulphate was prepared by the addition of an equivalent of dimethyl sulphate, freshly distilled *in vacuo*, to phenazine in nitrobenzene solution [Kehrmann, 1913; Hillemann, 1938] and crystallized quickly from alcohol, M.P. 167°. Methylphenazonium chloride was obtained as yellow needles by repeated crystallization of the methosulphate in presence of KCl (found: Cl 15·1; calc. for $C_{13}H_{11}N_2Cl$ 15·4%). This salt has previously been described as green by Browning *et al.* [1922] who prepared it by dissolving in HCl the product obtained by action of alkalis on phenazine methosulphate. This must be regarded as impure, for methylphenazonium salts decompose in alkaline solutions [McIlwain, 1937, 2; Hillemann, 1938].

Methylphenazonium phosphate was prepared by the addition of excess Na_2HPO_4 to the methosulphate, each in the minimum amount of water; the salt separated as brown prisms.

Semiquinoid methylphenazonium phosphate was obtained in fine green needles by warming the methosulphate in alcohol with excess phosphoric acid: I_2 equiv. [McIlwain, 1937, 2] 299; calc. for $C_{13}H_{11}N_2PO_4$ 292. Partial reduction of the methylphenazonium salts under warm acid conditions has been previously recorded [Kehrmann, 1914].

2-Aminophenazine methosulphate was prepared according to Kehrmann [1913] and the remainder of the compounds by the methods previously described [McIlwain, 1937, 1, 2].

Potentiometric titrations

The potentials were determined by titration of $M/2000$–4000 solutions in phosphate buffer with *c. M/200* chromous acetate, prepared by diluting the satd. aqueous soln. with 2 vol. de-aerated water in a storage burette of the type described by Cohen & Phillips [1929]. The electrode vessel was kept at $30 \pm 0.1°$ and de-aerated and stirred with N_2 freed from O_2 by hot Cu. Potentials were read from 3 gold-plated electrodes which normally agreed within 1 mv. Quinhydrone in 0.1 N HCl was used as the standard half-cell.

The titration curves showed no evidence of 2-stage oxidation-reduction processes over the pH range investigated, but slight intermediate colours were in some cases observed. The initially yellow solutions of the alkyl phenazonium salts become slightly yellow-green before separation of the colourless dihydrocompound on complete reduction. The reduced keto compound was pale cream coloured and insoluble; the reduced sulphonates and aminophenazine were soluble and almost colourless.

$Na_2S_2O_4$ was initially used as the reducing agent but though this gave normal titration curves, the results were not considered reliable as the titration could not be repeated after oxidation of the reduced solution. Progressively more positive values were obtained on repetition. A secondary reaction had evidently occurred as the solution showed a green fluorescence and the reduced compounds were no longer insoluble. In the case of phenazine methosulphate the values approached those of the sulphonates. It is understandable that such sulphonates should be produced from the sulphite formed during reduction, as their preparation from phenazonium salts and sulphite has been described [McIlwain, 1937, 2]. The initial values in titration with $Na_2S_2O_4$ were however substantially correct; Preisler & Hempelmann [1937] have used $Na_2S_2O_4$ in such titrations.

The compounds investigated considerably increase the range of oxidation-reduction potentials observed in the phenazine series (Table I). The simple

Table I

Compound	E_h, pH 7 30	Authority
Na N-methylphenazoniumdisulphonate betaine	$+0.230$	Present investigations
N-Methylphenazonium sulphonic acid betaine	$+0.130$	,,
Phenazine methosulphate	$+0.080$	,,
Phenazine ethosulphate	$+0.055$	,,
4-Keto-N-methylphenazine (pyocyanine)	-0.034	[Friedheim & Michaelis, 1931]
4-Keto-N-ethylphenazine	-0.055	Present investigations
Phenazine-1-carboxylic acid amide (chlororaphin)	-0.115	[Elema, 1933]
2-Amino-N-methylphenazine methosulphate	0.145	Present investigations
2-Keto-N-methylphenazine	-0.165	[Preisler & Hempelmann, 1937]
1-Hydroxyphenazine	-0.173	[Michaelis, 1931]
2-Hydroxyphenazine	0.214	[Preisler & Hempelmann, 1937]
2:7-Diamino-N-phenylphenazonium chloride (phenosafranine)	-0.252	[Stiehler *et al.* 1933]
2-Amino-7-dimethylamino-2-methylphenazine (neutral red)	-0.325	[Clark & Perkins, 1932]

phenazine quaternary salts and sulphonic acids are much more positive than previously studied compounds, some examples of which are quoted. Table 1 also affords an excellent example of correlation of E_h with the electronic properties of the substituent groups, electron-attracting groups producing more positive and electron-repelling groups more negative potentials.

Phenazines as carriers

Methods. The O_2 uptake was measured in Warburg manometers at 37·5°. Each vessel contained in the main part 2 ml. enzyme solution, 0·1 mg. coenzyme of purity 0·2 (in some experiments 0·02 mg. of purity 1), 0·3 ml. $M/10$ hexosemonophosphate. The side bulb held the solution of carrier in 0·2 ml. The inner cup contained 0·2 ml. N NaOH and the gas space was filled with pure O_2. Usually these conditions were adhered to, but sometimes slight variations were made. The O_2 uptake during the first hr. was measured; it continued regularly for a longer period provided that the substrate concentration did not fall too much. Readings were corrected for a blank without substrate. In the absence of added substrate the blank was small, a few μl. per hr., except when large quantities of autoxidizable carrier were present. This autoxidation is much reduced by working at a slightly acid reaction, pH c. 6·5, without affecting the catalytic activity, and this was done in later experiments; other experiments were at pH 7–7·5. The rates of O_2 uptakes were the same with the various combinations of enzyme and coenzyme used, within fairly close limits, since both these components were present in excess and the amount of carrier was ordinarily the limiting factor. The carriers were dissolved in water and where necessary neutralized. A few (Table II, Nos. 12, 19) were sparingly soluble and a suspension containing the wt. shown was placed in the side bulb. Flavin enzyme was dissolved in 10 vol. of water and centrifuged to remove a trace of denatured protein. Amounts of carrier are expressed in mg. and μl. ($1 mM = 22400 \mu$l.). In the case of flavin enzyme $1 mM$ prosthetic group, photometrically determined, is taken as 22400μl.

Results. These are shown in Table II.

The results may be grouped according to the rate of O_2 uptake.

Group 1. Maximum activity (c. 200μl./hr.) is given by only a few substances. These were: phenazine methosulphate, methochloride and ethosulphate (amount of carrier c. 20μl.) and flavin enzyme (Nos. 3, 4, 5 & 10).

Group 2. Of less but still marked activity were the mono- and di-sulphonic acid derivatives of N-methylphenazine (Nos. 1 and 2), pyocyanine (No. 8) and its ethyl homologue (No. 9) whose activity exceeds that of pyocyanine, the 2-amino derivative of N-methylphenazine methosulphate (No. 14) and the red oxidation product of N-methylphenazine quaternary salts (No. 15) and its ethyl homologue (No. 16). In amounts of from 22–50μl. this group caused O_2 uptakes of from 40–80μl./hr. It is noteworthy that all the compounds of Groups 1 and 2, with the exception of the flavin enzyme, are derivatives of N-alkylphenazines.

Group 3. Among non-phenazine compounds investigated none approached in activity the simple phenazine quaternary salts. Brilliant cresyl blue and methylene blue showed most activity in this group, being comparable with the lower members of Group 2 (50μl. dye caused c. 30–45μl. O_2/hr.).

Group 4. The remaining compounds studied were of low or zero activity (with chlororaphin and dimethylalloxazine the lack of activity might have been due to their insolubility). Methyl Capri blue, though in the same region of potential as active carriers, belongs to this group. Acridine methochloride (No. 22, Table II) was quite inactive as carrier, though chemically it differs from phenazine methochloride only in the substitution of a CH group for a N atom.

Table II

No.	E_h, pH 7	Substance	Amount per vessel		O_2 uptake*	Turnover no. (Average over 1st hr.) $\dfrac{\text{Mol. } O_2/\text{min.}}{\text{Mol. catalyst} \times 0.5}$
			mg.	μl.	μl. (1st hr.)	
1	+0·230	Sodium N-methylphenazonium disulphonate betaine	0·5	30	79	0·088
2	+0·130	N-Methylphenazonium sulphonic acid betaine	0·5	37	68	0·061
3	+0·080	Phenazine methosulphate	0·3	22	204	0·310
4	--	Phenazine methochloride†	0·3	27	206	0·260
5	+0·055	Phenazine ethosulphate	0·3	19	190	0·330
6	+0·045	Brilliant cresyl blue	1·5	106	71·5	0·022
			0·75	53	47	0·029
			0·5	35	36	0·034
7	+0·011	Methylene blue	1·5	90	34	0·013
			1·0	60	28	0·016
			0·75	45	27	0·021
			0·5	30	27	0·030
			0·25	15	26	0·057
8	-0·011	Pyocyanine hydrochloride† (4-keto-N-methylphenazine)	0·45	45	47	0·030
9	-0·055	4-Keto-N-ethylphenazine	0·3	30	80	0·090
10	0·060	Flavin enzyme	(20)‡	0·18	215	39·600
			(5)‡	0·045	110	81·400
11	0·060	Methyl Capri blue	0·3	22	13	0·020
12	-0·115	Chlororaphin (satd.)	~0·3	<30	15	<0·020
13	-0·142	Gallophenin	0·5	37	4	0·003
14	0·145	2-Amino-N-methylphenazine methosulphate	0·3	21	40	0·063
15	-0·165	2-Keto-N-methylphenazine	0·5	50	69	0·047
			0·14	14	35	0·083
16	0·180	2-Keto-N-ethylphenazine	0·3	28	45	0·053
17	-0·180	Lactoflavin	0·3	20	0·5	0·001
18	0·252	2:7-Diamino-N-phenylphenazonium chloride (phenosafranine)	0·3	21	0	0·000
Other compounds:						
19	—	6:7-Dimethyl alloxazine (satd.)	<0·1	<7	0	0·000
20	--	Nicotinamide methiodide	0·5	42	1	0·000
21	—	Phenazine-1-carboxylic acid amide	0·5	49	4	0·003
22	·	Acridine methochloride	0·3	29	0	0·000
23	—	1:2:3:4-Tetrahydrophenazine methosulphate	0·3	20	25	0·040
24	--	Aneurin	2·0	133	0	0·000
25	—	Thiochrome	0·6	51	0	0·000

* Corrected for blank, if any.   † See Fig. 1.
‡ Wt. of crude substance taken; flavin content photometrically determined.

The catalytic effect is dependent on the concentration of carrier. This is clearly shown by the data of Table II for brilliant cresyl blue and methylene blue. The turnover no. in the last column shows the decreasing activity with increasing concentration of these dyes. Since this is not here due to the limiting capacity of the enzyme system, both being feeble catalysts for this system, it is apparently due to a poisoning effect of these dyes when present in high concentration.

A different effect is seen with the N-alkylphenazines. Here, owing to their greater activity, the O_2 uptake is limited by the enzyme system when the amount of these catalysts is much increased. Fig. 1 shows the activity-concentration curves of phenazine methochloride and pyocyanine. (The preparation of enzyme was actually made to study the oxidation of phosphohexonate, the curves for

which are also included for comparison in Fig. 1. The rather high concentration, $M/30$, of phosphate buffer has inhibited the hexosemonophosphate oxidation more than that of phosphohexonate [see Dickens, 1938].)
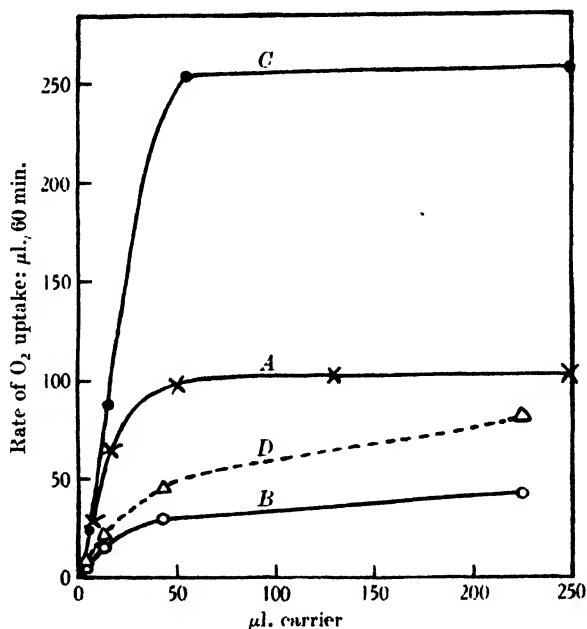
Fig. 1. Comparison of phenazine methochloride and pyocyanine as carriers. Curve A: phenazine methochloride, hexosemonophosphate. Curve B: pyocyanine, hexosemonophosphate. Curve C: phenazine methochloride, phosphohexonate. Curve D: pyocyanine, phosphohexonate. 20 mg. acetate-precipitated enzyme, 2 ml. $M/30$ phosphate buffer, 0·1 mg. coenzyme (purity 0·2), 0·3 ml. substrate.

Activity relative to flavin enzyme

While the absolute values of O_2 uptake with the hexosemonophosphate system when phenazines are added attain those given by flavin enzyme as carrier, the turnover numbers (T)[1] in Table II show clearly that their efficiency is very much less. For the flavin enzyme, $T = 40$–80 mol. $O_2/0.5$ mol. prosthetic group min. with our enzyme preparation. Warburg & Christian [1933] give the values as 25–50 mol. O_2/mol. prosthetic group min. In contrast with this high activity, the non-colloidal carriers listed in Table II give much lower T-values, the most active phenazonium salts having only about 1/250th of that of the flavin enzyme, while pyocyanine in similar concentration is about ten times less active still.

E_h relationships: "specificity"

The E_h of the simple N-alkylphenazonium salts varies from 0·08 to 0·06. That of the flavin enzyme is recorded by Kuhn & Boulanger [1936] as -0.06 V. (i.e. 0·12 V. more positive than lactoflavin). Although intermediate between

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[1] For the calculation of the turnover no. it is important to know if the O_2 taken up is partly lost in side reactions (e.g. H_2O_2 formation or coupled oxidation) or if it is wholly reduced by the H transported by the catalyst concerned. Only in the latter case is the turnover no. given by the ratio: mol. $O_2/0.5$ mol. catalyst min. With 100% yield of H_2O_2 the equation becomes $T = $ mol. O_2/mol. catalyst min., as used by Warburg & Christian [1933].

these in potential methylene blue and brilliant cresyl blue are inferior catalysts for this reaction. Hence thermodynamic considerations alone do not govern the efficiency of the carriers tested in this system. It is also noteworthy that other phenazines (Nos. 1, 2, 14, 16, Table II) whose potentials lie outside those of the alkylphenazonium salts and flavin enzyme in both positive and negative directions, are somewhat more efficient catalysts than the dyes methylene blue and cresyl blue, although according to their potentials these dyes should have activities between that of the most active alkylphenazines and flavin enzyme. There is thus a certain limited "specificity" in the chemical nature of the active carriers for this system. It may be possible to explain this structural specificity in terms of semiquinone formation [Michaelis *et al.* 1936, 1, 2]. The activity may be determined by the concentration of semiquinone in the half-reduced form at the reaction of the system.

Catalytic activities of phenazines added as semiquinones

Semiquinonoid methylphenazonium phosphate and 1:2:3:4-tetrahydrophenazine methosulphate [McIlwain, 1937, 1] were tested. The activity of the semiquinonoid and ordinary forms of the compounds was the same, but as the bright green solution of semiquinones rapidly changed to yellow on addition to the enzyme system, it is evident that only a trace of semiquinonoid compound could have been present, and this of course is no evidence against the importance of semiquinone formation.

Qualitative differences in effect of phenazines and flavin enzyme

Whereas the enzyme system used attacked Neuberg & Robison esters at about the same rate in presence of flavin enzyme as carrier, the Neuberg ester was much
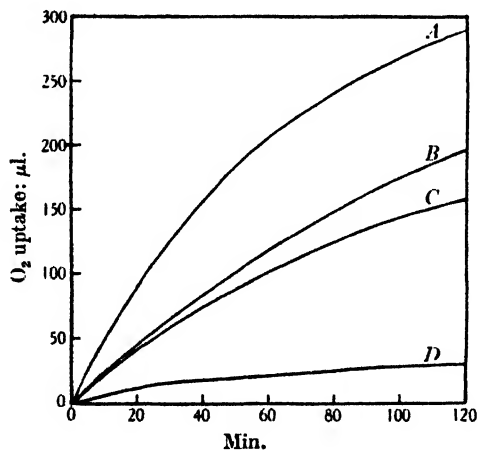
Fig. 2. Curve A: Robison ester + 0·3 mg. phenazine methochloride, 0·3 mg. phenazine methosulphate, or 10 mg. (crude) flavin enzyme. Curve B: Neuberg ester + 5 mg. flavin enzyme. Curve C: Robison ester + 5 mg. flavin enzyme. Curve D: Neuberg ester + 0·3 mg. phenazine methochloride. 0·1 mg. Zwischenferment in 2·3 ml. $M/100$ phosphate buffer, + 0·02 mg. coenzyme II of purity 1, + 0·4 ml. $M/6$ phosphoric ester.

less rapidly attacked than the Robison ester, when phenazine methochloride was used instead of flavin enzyme (Fig. 2).

Neuberg ester was prepared from Ca hexosediphosphate (B.D.H.) by hydrolysis with oxalic acid [Neuberg, 1918]. The Ca salt thus obtained was decomposed by the calc. amount of oxalic acid and after removal of Ca oxalate neutralized with NaOH.

As yet the mechanism of the oxidation of Neuberg ester has not been examined. It is therefore difficult to explain this difference, which may be accounted for either by the presence of some other enzyme than the flavin one in the crude preparation of flavin enzyme, or less probably by some inhibitory action of the phenazine compound on a component of the fructosemonophosphate-oxidizing system. That phenazine compounds can exert an inhibitory action on enzymes is readily shown. Weil-Malherbe [1937, 1] showed that succinic dehydrogenase is inhibited by pyocyanine and phenosafranine. The action of phenazine methochloride on carboxylase and succinic dehydrogenase is shown in Table III.

Table III. *Inhibition of carboxylase and succinic dehydrogenase*

Carboxylase. 50 mg. yeast carboxylase [Axmacher & Bergstermann, 1934] in 0·9 ml. water per vessel, with 0·3 ml. phosphate buffer pH 6·5 and 1 ml. addition of dye (or water). Side bulb contained 0·2 ml. $M/10$ Na pyruvate. Gas space, N_2. Temp. 25 .

Addition:	Water	2 mg. pyocyanine	2 mg. phenazine methochloride	2·6 mg. phenosafranine	2·5 mg. cresyl blue	1·7 mg. 2-keto-N-methyl-phenazine
			CO_2 evolved (μl.)			
30 min.	195	192	95	154	182	192
60 ,,	228	223	112·5	198·5	216·5	235
120 ,,	250	242	121·5	232	242	259

Inhibition by $M/300$ phenazine methochloride $= 50\%$; others, none.

Succinic dehydrogenase. 50 mg. muscle enzyme, suspended in 1·5 ml. $M/10$ succinate in $M/50$ phosphate buffer pH 7·4; 0·5 ml. cytochrome. Side bulb contained dyes 0·4 ml. $M/50$. Air, 37·5 ; 0·2 N NaOH in inner cup. Phenazine methochloride readings corrected for autoxidation of carrier.

Addition	Water	Phenazine methochloride	Pyocyanine	Phenosafranine
O_2 uptake μl. (60–120 min.)	98	76	32	28
Inhibition %	—	23	68	70

In these two cases very different actions are seen; phenazine methochloride in $M/300$ concentration inhibits carboxylase by 50 % but has little action on succinic dehydrogenase. On the other hand pyocyanine and phenosafranine, which strongly inhibit succinic dehydrogenase [Weil-Malherbe, 1937, 1] have little effect on carboxylase. Michaelis & Smythe [1936] have already drawn attention to the poisoning by a similar concentration of pyocyanine of glucose fermentation in Lebedew fluid and its restoration by addition of a carboxylase preparation.

Action of iodide ion

In the earlier experiments with this system we used phenazine methiodide as in the earlier tissue experiments [Dickens, 1936, 1]. It was found with the hexosemonophosphate system that, using the methiodide as carrier, the reaction came to a standstill after 20–40 min. This was due to the I⁻ of the quaternary salt, since it did not occur with phenazine methochloride and the action of phenazine methochloride and flavin enzyme is inhibited by small quantities of NaI (Fig. 3).

The inhibition is perhaps due to an interaction either of I^- or of I_2 with the coenzyme, for it appeared to be partially removed by addition of fresh coenzyme to the system. The inhibition by I^- does not appear to have been observed before.
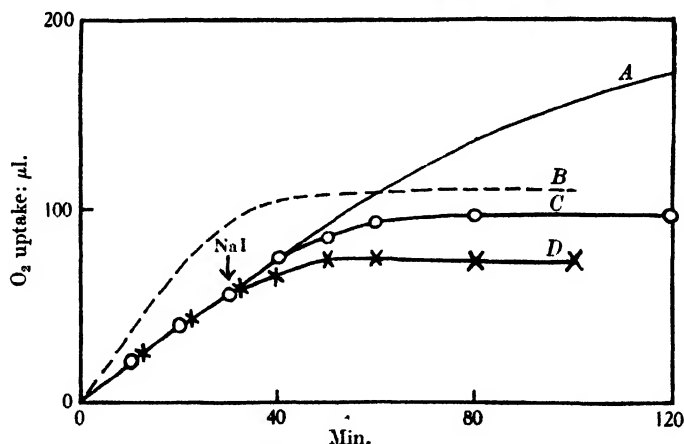
Fig. 3. Hexosemonophosphate oxidation. Curve A: 0·15 mg. methylphenazonium salt* or 5 mg. flavin. Curve B: 0·16 mg. phenazine methiodide. Curve C: 5 mg. flavin enzyme + 0·1 mg. NaI, 2H₂O tipped in at 30 min. Curve D: 0·15 mg. methylphenazonium salt* + 0·3 mg. NaI, 2H₂O tipped in at 30 min.

Action of phenazine methochloride on tissue metabolism

In view of the above results the action of the methochloride on tissue slices was tested. The results do not differ greatly from those seen with the methiodide [Dickens, 1936, 1], but as the fall of anaerobic glycolysis is less with the methochloride, the apparent partial restoration of the Pasteur mechanism is better seen (Table IV). The results with the red oxidation product of N-methylphenazines (2-keto-N-methylphenazine) were obtained with a pure specimen made synthetically [Kehrmann, 1924], and not by autoxidation, since free phenazine always results by the latter method; the specimen used was freely soluble in

Table IV. Action of phenazine methochloride and 2-keto-N-methylphenazine on metabolism of Jensen sarcoma slices

Warburg two-vessel technique, NaHCO₃-glucose Ringer. Temp. 37·5°. CrCl₂ in anaerobic vessels.

	Min.	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$
			Control			Phenazine methochloride ($10^{-3} M$)	
Exp. 1	0–60	−10·8	+22·2	+40·8	−13·5	+ 8·6	+45·6 (yellow)
	60–120	—	—	+36·4	—	—	+31·9 (colourless)
	120–180	—	—	+35·0	—	—	+25·0 (colourless)
Exp. 2	0–30	−14·3	+21·4	—	−28·0	+12·1	—
	30–60	−10·1	+18·6	—	−10·3	+ 4·1	—
	60–90	− 9·1	+18·0	—	− 9·6	+ 3·1	—
			Control			2-Keto-N-methylphenazine ($10^{-3} M$)	
Exp. 3	0–60	−16·8	+20·4	+28·2	−18·3	+13·8	+16·6 (red)
	60–120	−14·7	+20·0	+17·5	−15·7	+14·0	+13·5 (v. pale pink)
	120–180	−13·1	+15·5	+15·9	−15·1	+13·8	+13·7 (colourless, re-oxidized to red on admitting air)

* Not the iodide.

salt solution unlike that previously employed [Dickens, 1936, 1] which was there-fore impure. In aerobic experiments with tissue the phenazonium salts become partly oxidized to a red compound, presumably this one. This compound inhibited anaerobic glycolysis more than phenazine methochloride, but did not reduce the aerobic glycolysis as much in Exp. 3 of Table IV, though it had a pyocyanine-like action on aerobic glycolysis.

Autoxidation. Measured in $NaHCO_3$-glucose Ringer solution, by the two-vessel method of Warburg under the same conditions as in the tissue experiments, the autoxidation of 10^{-3} M phenazine methochloride in 95 % O_2 + 5 % CO_2 was slow, $-3\,\mu l.$ O_2, $+4\,\mu l.$ CO_2 per 4 ml. per hr. When tissue is present the oxidation is probably greater, but it is difficult to allow for it accurately. Calculations showed that with the quantities used, it could not result in any serious error, and that the measurements were substantially those of tissue metabolism and not of autoxi-dation of the carrier. When in experiments without tissue, in which autoxidation was allowed to proceed in a more alkaline medium, the O_2 consumption was measured and the acid production was titrated, it was found that for 1 mol. phenazine methochloride 0·5 mol. O_2 is consumed and 1 equiv. acid (1 mol. HCl) is set free. Hence autoxidation would be expected to increase aerobic acid production, whereas the addition of phenazine methochloride to tissue slices produces a fall of aerobic glycolysis and it is in fact probable that part of the residual acid production in presence of phenazine methochloride arises from this. On the other hand reduction anaerobically may have given values for glycolysis which were a little too high, but calculation showed a max. error of c. 10 %.

4·6 mg. phenazine methochloride were added from the side bulb of a Warburg manometer vessel to 4 ml. $N/100$ NaOH contained in the main part. Air, 37·5°. The initially rapid oxidation became very slow after 50 min. when 199 $\mu l.$ O_2 had been consumed and the equiv. of 1·80 ml. $N/100$ acid liberated = 403 $\mu l.$ Calc. for $\frac{1}{2}O_2$ and 1HCl; 224 $\mu l.$, 448 $\mu l.$ respectively. A bright red $CHCl_3$-soluble compound resulted. Indophenoloxidase preparation from heart muscle brings about oxidation of phenazine methochloride at neutral reaction to form a red compound soluble in $CHCl_3$. The quantitative investigation of this has not been made. Free phenazine in addition to the red compound results from alkaline oxidation of the methochloride [McIlwain, 1937, 2].

SUMMARY

The E_h of a series of phenazine derivatives, determined by potentiometric titration, have been correlated with the electronic properties of the substituents. The activity of the phenazines and some non-phenazine dyestuffs as carriers in the hexosemonophosphate system has been determined. According to their activity, the substances fall into four groups: (1) most active: phenazine metho-sulphate, methochloride and ethosulphate, E_h +0·055 to +0·080; (2) moderately active: mono- and di-sulphonic acids derived from N-methylphenazine, E_h +0·13, +0·23; pyocyanine, E_h −0·011 and its ethyl homologue; (3) less active: brilliant cresyl blue and methylene blue; (4) others inactive: the most active of the above compounds had only about 1/250th of the turnover no. of the flavin enzyme, E_h −0·06. Structure is evidently more important than E_h in determining the activity of carriers for this system. A property common to the phenazines and flavin is the ability to form semiquinones, and the peculiar activity of these compounds in this system may be due to this property.

When phenazine methochloride is used instead of flavin enzyme, the Neuberg ester is attacked much less readily than the Robison ester; with flavin enzyme as carrier both are attacked at about the same rate. Carboxylase is inhibited by $M/300$ phenazine methochloride, not by pyocyanine or phenosafranine; succinic

dehydrogenase is inhibited by phenosafranine, but much less by phenazine methochloride in the concentration used. The hexosemonophosphate system is inhibited by iodide in low concentration. The action of phenazine methochloride on the metabolism of tumour tissue is to increase respiration and diminish aerobic glycolysis.

We wish to thank Prof. O. Warburg for generous gifts of purified enzyme and coenzyme, and Prof. R. Robison for the analysis of our hexose monophosphate. We are much indebted to Dr B. C. J. G. Knight for help and advice in connexion with the E_h measurements.

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CCXI. OXIDATION OF PHOSPHOHEXONATE AND PENTOSE PHOSPHORIC ACIDS BY YEAST ENZYMES

I. OXIDATION OF PHOSPHOHEXONATE

II. OXIDATION OF PENTOSE PHOSPHORIC ACIDS

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IN a preliminary note [Dickens, 1936] a scheme of oxidation of carbohydrate was outlined in which the first stages suggested were the esterification of the hexose to hexosemonophosphate, oxidation of this first to the phosphohexonate, then to the 2-ketophosphohexonate, decarboxylation to a pentose phosphoric acid and by a continuation of the process finally pyruvic acid, which would be completely combusted.

At that time the evidence in favour of this scheme was slight. No proof of the formation of pentose phosphoric acids by oxidation of hexosemonophosphate or of phosphohexonate existed. The theory of the formation of 2-ketophosphohexonic acid was first advanced by Lipmann [1936] on the basis of O_2 uptake experiments only. Lipmann found that phosphohexonic acid was oxidized by yeast macerate, and that for each mol. phosphohexonate $\frac{1}{2}O_2$ was consumed and 1–1·5 CO_2 appeared, the CO_2 being only about 1 mol. in presence of bromoacetate. On the basis of this evidence, Lipmann advanced the theory that 2-ketogluconic acid was first formed ($\frac{1}{2}O_2$ consumed), this was then decarboxylated ($1CO_2$ liberated) and the further process consisted in fermentation of the theoretically expected d-arabinosemonophosphate, this stage being the one inhibited by bromoacetate. Some evidence tending to show keto-acid formation was given by experiments [Dickens, 1936] in which HCN appeared to act as ketone fixative, and the addition of a preparation of carboxylase to a carboxylase-free enzyme preparation caused a further oxidation and CO_2 evolution with phosphohexonate.

None of these results, however, was sufficiently clear to provide a basis for this theory. Warburg & Christian [1936, 1] showed, by using a more active enzyme preparation, that the "end-point" in Lipmann's experiments was only apparent and was in reality due to a gradual destruction of the activity of the enzyme. The experiments with HCN and carboxylase [Dickens, 1936] were too indirect. The critical points which had to be shown were:

1. Pentose phosphoric acids must be shown to arise from the oxidation and decarboxylation of hexose phosphoric acids.

2. It must be shown that pentose phosphoric acids are readily oxidized and fermented by yeast extracts.

Both these points are established in the work to be described. In the first part of this paper the oxidation of phosphohexonic acid is described, and confirmation of the occurrence of 5-C phosphoric esters giving the pentose reaction [Warburg & Christian, 1937] is obtained. Further oxidation of the phosphohexonic acid to a

4-C phosphoric ester is shown to occur. In the second part, it is shown that pentose phosphoric acid can be readily oxidized by yeast enzyme in the presence of coenzyme II.

In the subsequent paper [Dickens, 1938] it will be shown that pentose phosphoric acid is readily fermentable by yeast enzymes in the presence of coenzyme I and inorganic phosphate.

During this work, the important point arose that the pentose phosphoric acid most readily attacked both oxidatively and by fermentation, is not the d-arabinose-5-phosphoric acid which would be expected to arise from simple 2-oxidation and decarboxylation of d-glucose, but is the stereoisomeride d-ribose-5-phosphoric acid. Since d-ribose is the naturally occurring pentose of animal and yeast cells, this fact is of great physiological interest, and may perhaps give a key to the metabolism of pentoses in living cells. d-Ribose is not itself fermentable, either by yeast cells or yeast extract, nor is it oxidized by brain slices or by yeast extract. Apparently direct phosphorylation of ribose presents difficulty in these materials, and the theory is advanced that d-ribose-5-phosphoric acid may originate from hexoses by a process of phosphorylation, oxidation and decarboxylation. Indirect evidence is presented in these papers which makes this assumption probable, and direct evidence will be sought in future experiments.

I. OXIDATION OF PHOSPHOHEXONATE

Enzyme. Prep. A. The enzyme preparation used in most experiments was made from Lebedew yeast maceration fluid by the method of acetate precipitation already described [Dickens & McIlwain, 1938] and the general methods for experiment were the same as detailed there, except that phosphohexonate was used as substrate. During the course of this work we have used three different yeasts: (1) Newcastle Brewery Co. top fermentation yeast. (2) Messrs Tennent, Glasgow, bottom yeast. (3) Löwenbräu, Munich, bottom lager beer yeast. Although there was little difference in the activities of the macerates from these, successful precipitation of enzyme without excessive dilution of the Lebedew juice was obtained only with the German yeast, presumably owing to its lower content of protective hop resins, which held up the precipitate with the British yeasts.

Prep. B. In earlier experiments a preparation was made from Tennent's yeast Lebedew juice by diluting 50 times with distilled water, passing CO_2, centrifuging from the inactive precipitate after 10 min., passing CO_2 to saturation and keeping in a stoppered bottle in the refrigerator overnight [cf. Warburg & Christian, 1933]. The centrifuged precipitate, dried *in vacuo* over H_2SO_4 and KOH was used: yield c. 250 mg. per 100 g. dried yeast; 5–20 mg./vessel were needed. This preparation attacked hexosemonophosphate more rapidly than phosphohexonate; otherwise there was little difference between A and B, but both differ from "Proteins I and II" of Warburg & Christian [1937] in that the precipitated enzyme is washed with acid in our two preparations, and this may account for some differences between the results of Warburg & Christian and those to be described.

The activities of the original Lebedew juice and of Preps. A and B are shown in Table I. It is evident that much activity is lost in the precipitation, and this passes into the acid supernatant liquid, from which Warburg & Christian [1937] prepare their enzyme. This loss is compensated for to some extent by the simplicity of the method and by the greater stability of the acid-precipitated enzyme.

Table I. *Activities of original Lebedew juice and Preps. A and B*

All experiments at 38°, O_2, 0·2 ml. KOH in inner cup.

Original Lebedew juice. Dissolved to 2·2 ml. with $M/100$ phosphate containing 0·1 mg. coenz. II, 0·3 ml. $M/10$ substrate, and 0·2 ml. $M/100$ phenazine methochloride.

			μl. O_2 consumed per hr. with substrate	
Source	Per vessel ml.		Hexosemono-phosphate	Phospho-hexonate
Newcastle Brewery Co.	0·20	34	226	287
	0·05	4	56	63
Löwenbräu, Munich	0·20	—	225	311
	0·05	—	63·5	141

Acetate precipitates (A). Dissolved in $M/50$ phosphate of pH 7·4, pH c. 6·5. 0·1 mg. coenz. II; 0·3 ml. $M/10$ substrate; 0·2 ml. $M/100$ phenazine methochloride.

Source	Mg.	Corrected for blank (c. 10 μl. hr.)	
Löwenbräu, Munich	5	55	50
	10	90	90
	20	121	141
	50	155	180
CO_2 *precipitate (B):*			
Tennent, Glasgow	5	207	42

Numerous attempts to prepare stable extracts from the acid supernatant liquid proved unsuccessful: Warburg & Christian [1937] have since overcome this difficulty by warming the ½-sat. ammonium sulphate solution of the enzyme to 50°, thus "stabilizing" it, but the work described in this section was done before the publication of this method. Presumably destructive enzymes are removed by both methods.

Phosphohexonate. In a few earlier experiments, but not in any of the isolation experiments, the phosphohexonate was made from the prep. of muscle hexose-monophosphate described in the preceding paper [Dickens & McIlwain, 1938] by the method of Robison & King [1931]. Later Prof. Robison kindly gave a prep. of the neutral Ba salt of 6-phosphogluconate containing some 6-phosphoman-nonate with < 1·5 % of reducing hexosephosphate. Analysis (Prof. Robison): H_2O, 7·0 %. Found (dry subs.): total P, 6·34 %; inorg. P, 0·06 %; H. and J. titn. with added NaOH, 0·5; $[\alpha]_{5461} - 1·2°$. Calc. for $(C_6H_{10}O_{10}P)_2Ba$: P, 6·42 %.

The solution of Na salt was prepared by grinding the Ba salt (288 mg.) with a slight excess of Na_2SO_4, $10H_2O$ (295 mg.) and centrifuging from Ba_2SO_4 after making up to 6 ml. with water and addition of 0·055 ml. N HCl. The concentration of ester P was estimated on this solution (c. 0·09 M).

Coenzyme. Coenzyme II of purity 1 was given by Prof. Warburg. Coenzyme II of purity 0·2 was prepared from horse blood corpuscles [Warburg & Christian, 1936, 2] and its activity checked by comparison with the Warburg preparation: yield 80 mg. from 2·2 l. washed corpuscles. This preparation contains also coenzyme I and an adenine nucleotide [Warburg & Christian, 1936, 2]. Usually 0·02 mg. of purity 1, or 0·1 mg. of purity 0·2 was used per vessel. Unlike the hexosemonophosphate oxidation, that of phosphohexonate is not always maximal with these amounts, and in some "end-point" experiments they were increased fourfold. Possible reasons for this are indicated later.

Carriers. Flavin enzyme and phenazine methochloride were used as previously [Dickens & McIlwain, 1938]. The O_2 uptake with phenazine methochloride was corrected for blank without substrate (autoxidation of phenazine metho-chloride + blank oxidation). The blank was small with Preps. A and B except in

a few "end-point" experiments where high concentrations of phenazine metho-
chloride were used to ensure a rapid reaction. With flavin enzyme the blank
was usually negligible.

Measurement of O_2 uptake. Warburg manometers filled with O_2 and containing
KOH in the inner cup were used at 37·5°. Where HCN was present it was added
as freshly prepared neutral solution, and instead of KOH, KCN-KOH mixture
[Krebs, 1935] was used to avoid absorption of HCN. CO_2 evolved was measured
(in absence of HCN) by a second vessel without KOH, which was acidified at the
end of the exp. to drive out bound CO_2, with a similar pair of vessels without
substrate as controls.

pH-activity curve. The titration curve of the enzyme (Prep. A, 20 mg./ml. in
$M/100$ phosphate buffer) was first determined and the contents of each vessel
after cooling in ice were adjusted to the required pH by addition of the required
vol. ice cold $N/100$ NaOH or, for the vessel at pH 4·7, $N/50$ acetic acid. Stronger
phosphate buffer cannot be used since it inhibits the reaction (see below). The
enzyme system with phenazine methochloride as carrier showed a broad pH
optimum at pH 6·3–7·5 (Fig. 1).
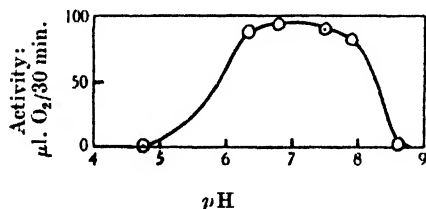
Fig. 1. pH optimum of phosphohexonic
system.
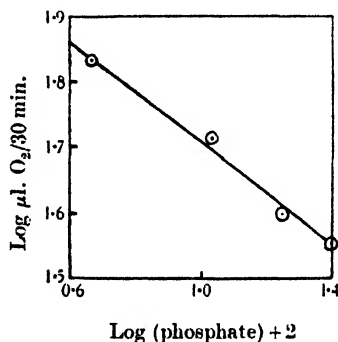
Fig. 2. Phosphate inhibition of
phosphohexonic system.

Phosphate inhibition. Conditions were as in the preceding exp. except that
pH was kept constant and varying concentrations of phosphate buffer were used.
During the first 30 min. of the exp. the inhibitions with 0·05, 0·1 and 0·25 M
phosphate were respectively 16, 32 and 57 %. Fig. 2 shows that, as with the
hexosemonophosphate system [Theorell, 1935], the velocity of oxidation varies
inversely as the phosphate concentration. Comparison with the data of Theorell
shows less inhibition for a given phosphate concentration with the phospho-
hexonic system than with the hexosemonophosphate one. This also is shown in
Fig. 1 of the preceding paper [Dickens & McIlwain, 1938].

Attempted separation of the phosphohexonic and hexosemonophosphate systems.
The above behaviour towards phosphate inhibition is typical of the slight
differences between these two systems, which makes their separation difficult.
As yet attempts to obtain an enzyme which attacks only phosphohexonate have
been uniformly unsuccessful, whether by dialysis, ammonium sulphate precipita-
tion, acetone precipitation or partial inactivation by incubation with alkali. On
the other hand, the "Zwischenferment" of Negelein & Gerischer [1936] is free
from the phosphohexonic enzyme [Dickens, 1936; Warburg & Christian, 1937].
It is probable that our enzymes, like those of Warburg & Christian [1937], are
mixtures of several components, as is shown by the different substrates attacked
and the multiple products formed.

Table II shows that the acid-precipitated enzymes require coenzyme II for their activity, purified cozymase being without effect. Gluconic acid is not oxidized unless phosphorylated, and the preparations contain no active carboxylase. An experiment with Warburg "Zwischenferment" is included to show its relative actions on hexosemonophosphate and phosphohexonate. In this exp. (Table II) the less pure sample of phosphohexonate was used, and the small O_2 uptake is probably due to traces of reducing hexosephosphate in the substrate.

Table II. *Action on various substrates: coenzyme required*

Enzyme in $M/100$ phosphate of pH 7·4; 0·04 mg. coenzyme II of purity 1; or 0·6 mg. purified cozymase; 0·5 ml. 0·8 N/HCN. Side bulb, 0·2 ml. 10% flavin enzyme. Inner cup, KCN-KOH. 0·4 ml. $M/10$ substrates. O_2. 37·5°.

Enzyme and coenzyme	Enzyme wt. per vessel mg.	Time min.	O_2 uptake (μl.) with substrate		
			Hexosemonophosph.	Phosphohexonate	Gluconic acid
Enz. B.:					
Coenz. I	8	30	—	− 2	0
		60	—	− 5	0
		90	—	− 6	—
Coenz. II	8	30	− 73·5	−49·5	− 0·5
		60	−125	− 78	−1
		90	−155	−101	—
Warburg "Zwischenferment":					
Coenz. II	0·1	30	−246	− 13·5	—
		60	−313	− 17·5	—

Enzyme B in $M/100$ KH$_2$PO$_4$, 0·2 ml. $M/5$ Na pyruvate, CO$_2$ evoln. (90 min.) + 2·5 μl.

Action of HCN. In the earlier experiments which were made with acid-precipitated enzyme and coenzyme II of purity 1, HCN (0·12 M) was necessary for the reaction to continue [Dickens, 1936]. This result (Fig. 3) was obtained many times when the pure coenzyme was used with small quantities of the enzymes A or B. By increasing the amount of enzyme, however, it was no longer apparent, the reaction continuing even in the absence of HCN. In the later work more care was taken to prevent destruction of enzyme during its precipitation and washing, by using ice cooling and quick manipulations, and it is possible that this may account for the difference between earlier and later preparations in their need for HCN. We are unable to account for the results otherwise.

End-point with pure coenzyme II. Table III shows experiments with and without HCN

Fig. 3. Effect of HCN on O_2 uptake with phosphohexonate (earlier prep. of enzyme).

for both hexosemonophosphate and phosphohexonate. In all these, coenzyme II of purity 1 was used; different end-points obtained with cruder coenzyme are described later.

The results of Table III show that under the conditions used, i.e. with pure coenzyme and vigorous enzyme preparations, the reaction proceeds either in presence or absence of HCN to an end-point which with phosphohexonate is

Table III. *End-point with pure coenzyme and acid-precipitated enzyme*

No.	ml. 0·09 M	μl.*	Enzyme prep. type	½	1	2	3	4	5	6	End-point. % of 1 mol. O₂ per mol. substrate
	Phosphohexonate or hexosemonophosphate			μl. O₂ uptake after hr.							
With HCN (0·12 M):											
Phosphohexonate											
1	0·4	810	A	130	222	329	360	380	—	390	48
2	0·2	402	B	56·5	122	181·5	—	—	—	- -	45
3	0·1	202	B	56	77	85	87·5	—	-	—	43
4	0·1	202	B	33	64	99·5	104	—	104	—	51
5	0·05	101	A	35	54	57·5	56	—	—	—	51
Hexosemonophosphate											
6	0·1	202	B	51	89	154	166	172	—	—	85
7	0·05	101	A	33	62	87·5	92	- - -	—	—	90
Without HCN:											
Phosphohexonate											
8	3·0	6050	A	—	—	—	—	—	—	3115	51·4
9	0·2	401	B	40·5	81·5	136·5	- -	157	—	167	42
10	0·1	202	B	37	61	78	98	107	—	—	53
11	0·05	101	B	31	43	53·5	64	66	—	—	65
Hexosemonophosphate											
12	0·1	202	B	51	89	166	172	—	—	—	85
13	0·05	101	B	—	87·5	101	117	—	—	—	115

* 1 mM. = 22400 μl.

close to that required for $\frac{1}{2}O_2$ per mol., with hexosemonophosphate $1O_2$ per mol. substrate. Although some very slow residual oxidation may remain, the figures of Table III, and still better the curves of these experiments, only one of which (Fig. 4) is reproduced, show the definite change in reaction rate at these points.
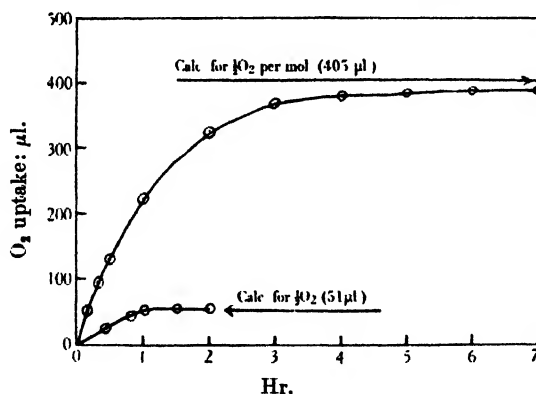
Fig. 4. End-point of phosphohexonic oxidation with coenzyme II of purity 1.

It seems most unlikely that this could be due in every case to destruction of enzyme coinciding with the different reaction times. Exp. No. 2, Table III, in which the enzyme with substrate and carrier, but without coenzyme, was incubated in the vessel for 1½ hr. at 37·5° before the coenzyme was tipped in, still gave a similar end-point corresponding to 90 % of the O₂ uptake required for $\frac{1}{2}O_2$ per mol. phosphohexonate.

The end-point observed corresponds fairly closely with the oxidation of both substrates to a stage corresponding with the formation of ketophosphohexonate.

H_2O_2 formation. H_2O_2 results from the autoxidation of the flavin enzyme when hexosemonophosphate is oxidized by the purified enzyme [Warburg & Christian, 1933]. H_2O_2 did not accumulate in the present experiments since (1) the acetate-precipitated enzyme contained very active catalase (25 mg. enzyme, 0·2 ml. 0·3 % H_2O_2, 114 μl. O_2/5 min.) (2) HCN did not affect the end-value although it would have inhibited the catalase. Accumulation of H_2O_2 would have changed the interpretation of the end-point, but the above experiments show that it did not occur.

Other ketone fixatives. The action of HCN which was seen with the earlier enzyme preparations, might have been due to its action as ketone-fixative, as in the experiments of Green & Brosteaux [1936] with lactic dehydrogenase. As these authors found for the lactic system, semicarbazide ($M/20$) and hydrazine hydrate ($M/8$) were without effect in the phosphohexonic system. Hydroxylamine and phenylhydrazine ($M/8$) caused irregular pressures; with sulphite and bisulphite no combination with keto-substance could be demonstrated by I_2 titration. Increases of O_2 uptake and CO_2 output equal to the O_2 consumed were regularly observed [Dickens, 1936] on the addition of crude carboxylase [Axmacher & Bergstermann, 1934], but this may be attributable perhaps to other enzymes or coenzymes contained in the carboxylase and should be repeated now that purified enzyme and cocarboxylase [Lohmann & Schuster, 1937] are available. Unphosphorylated 2-keto-*d*-gluconic acid is attacked only very slowly by active carboxylase (Table IV), but the phosphorylated compound may of course behave differently.

Table IV. *Action of yeast carboxylase*

50 mg. carboxylase prep., in 2 ml. $M/50$ phosphate buffer. Side bulb 0·1 ml. 2·5 N HCl. Air. 37·5°.

	μl. CO_2 evolved at			
	pH 7·4		pH 6	
	Addition: 0·2 ml. $M/10$ Na salt			
Min.	Pyruvate	2-Ketogluconate	Pyruvate	2-Ketogluconate
5	+ 30	0	+228	0
30	+152	0	+363	+ 5
60	+173	0	+391	+7·5
120	—	—	—	+9

Product from the oxidation of phosphohexonate with $\frac{1}{2}O_2$. A larger scale exp. was made to determine the nature of the product from the oxidation of phosphohexonate in presence of pure coenzyme II.

195 mg. enzyme A, well washed with acetate buffer [Dickens & McIlwain, 1938], were dissolved in 2·2 ml. water and the pH adjusted to 7·3 with ice cold $N/100$ NaOH (7·3 ml.). 0·3 mg. coenzyme II of purity 1 and 3 ml. 0·09 M Na phosphohexonate were added, the mixture was placed in two large Warburg manometer vessels containing 1·5 mg. phenazine methochloride and 0·5 ml. KOH in the side bulb and inner well of each vessel. O_2, 37·5°.

After 5 hr. the reaction became very slow and 3680 μl. O_2, corr. for autoxidation of the carrier, had been consumed. The oxidation thus went a little beyond the consumption (calc. 3050 μl.) of $\frac{1}{2}$ mol. O_2 per mol. phosphohexonate, perhaps because of traces of coenzyme contained in the large amount of enzyme used. The contents were precipitated with trichloroacetic acid and the supernatant liquid was neutralized with $Ba(OH)_2$, the resulting precipitate, together with that from addition to the mother liquor of 2 vol. abs. alc. was dried *in vacuo*; wt. 149 mg. This was placed in 5 ml. water, with $N/10$ HCl to make weakly acid. After centrifuging from the undissolved residue, the clear solution was neutralized with $Ba(OH)_2$ and was poured into 2 vol.

abs. alc. The precipitate ("Ba salt I") was well washed with 75% and abs. alc., then with ether and was dried *in vacuo* over P_2O_5; wt. 91 mg. = 60% of the amount of Ba phosphohexonate taken. Analysis (Weiler): Dried at 60° *in vacuo*, loss of wt. 7·26%. Found, for dry subs.: C, 15·50; H, 2·23; P (as phosphomolybdate) 7·04; Ba, 37·1%. Calc. for $C_5H_9O_9PBa$: C, 15·75; H, 2·36; P, 8·13; Ba, 36·1%. Calc. for $C_6H_{10}O_{11}PBa_{1·25}$: C, 15·62; H, 2·18; P, 6·73; Ba, 37·20%. The second formula is that of an acid Ba salt of 2-ketophosphohexonic acid; the first is that of the carboxylic acid derived from 2-ketophosphohexonic acid by oxidative decarboxylation, i.e. a phosphopentonic acid.

It seemed very probable that Ba salt I might be a mixture of these two compounds. Unfortunately their separation could not be achieved on the specimen available, and the repetition of the exp. has been left for a future occasion. An attempted purification by dissolving in HCl and making more alkaline than previously with $Ba(OH)_2$, resulted in a Ba salt containing much less P and Ba, these elements now being in the ratio 0·96:1. Found (Weiler): loss of wt. at 60° *in vacuo*, 5·02%. Dry subs. P, 5·23%; Ba, 24·4%. Some decomposition appeared to have occurred and the mixture could not be separated. Solution of a sample of the original Ba phosphohexonate in HCl and its reprecipitation by $Ba(OH)_2$ at similar alkalinity caused no such decomposition.

Oxidation of phosphohexonate in presence of coenzyme of purity 0·2: end-point and CO_2 formation. In the presence of the less pure coenzyme the oxidation with enzyme preps. A and B proceeds further than in the preceding experiments. Provided that sufficiently active enzyme is used in the presence of plenty of coenzyme, the O_2 uptake slightly exceeds 1 mol. O_2 per mol. phosphohexonate
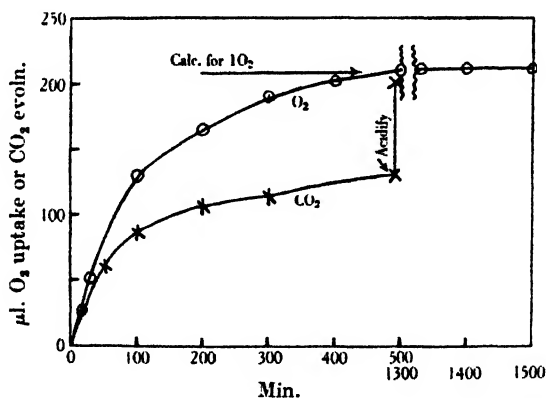
Fig. 5. End-point of phosphohexonic oxidation with coenzyme II of purity 0·2.

(Fig. 5) and the CO_2 formed is almost exactly equivalent to the O_2 uptake when the reaction has gone to completion. If however the reaction is stopped by acidification before the end-point is reached, the CO_2 formed then exceeds the O_2 consumed (Table V).

These results seem to indicate the occurrence during the earlier stages of the oxidation of an excess of decarboxylation over O_2 uptake. This could occur in two different ways: (1) initial stage; $\frac{1}{2}O_2$ consumed, keto-acid formed, keto-acid decarboxylated forming pentose phosphoric acid with evolution of 1 mol. CO_2; later slower oxidation by a similar type of mechanism of the pentose phosphoric acid formed. (2) Pentose phosphoric acid formed as in (1), but then undergoing cleavage with oxidation of the cleavage products formed.

Table V

Enzyme: 20 mg. per vessel Prep. A dissolved in 1·5 ml. $M/50$ phosphate buffer at pH 7·4, initial pH of mixture 6·22; 0·2 ml. = 0·1 mg. coenzyme of purity 0·2; 0·2 ml. $M/100$ phenazine methochloride in main part of vessel. Temp. 37·5°. O_2. Amount of substrate (from P estimation) = 404 μl./vessel.

Vessel no. ...	1	2	3	4
Enzyme, coenz., carrier, ml.	1·9	1·9	1·9	1·9
Bulbs:				
I. $M/10$ phosphohex., ml.	0·2	0·2	No substrate	
II. 2·5 N HCl, ml.	—	0·1	—	0·1
Inner cup, ml.	0·2 KOH	—	0·2 KOH	—
μl. O_2 or (free) CO_2 after min.	O_2	CO_2	O_2	CO_2
20	− 67·5	+ 60·5	− 0·5	+ 0·5
40	− 101	+ 93	− 0·5	+ 0·5
60	− 160	+ 147·5	− 1·5	+ 0·5
120	− 237	+ 206	− 4·5	+ 2
Total CO_2 on acidification	—	+ 329 uncorr.	—	+ 18·5
		+ 310·5 corr. for No. 4		

Hence when 0·58 mol. O_2 per mol. phosphohexonate has been consumed, the ratio $CO_2/O_2 = 1·31$.

At present it is not possible to decide between these alternatives, but it will be shown that both oxidation and fermentation of pentosemonophosphate can occur, and that a 4-C monocarboxylic-monophosphoric acid is formed oxidatively under conditions similar to those in the above exp.

Nature of the products formed from the oxidation of phosphohexonate in presence of crude coenzyme

Warburg & Christian [1937] have investigated the products formed when phosphohexonate is oxidized by flavin enzyme, pure coenzyme II and a yeast enzyme (prepared without washing with acid: "Protein of protein fraction I, 4th step") in the presence of methyl alcohol. This preparation differs from ours in its action. With our enzyme, and with phenazine methochloride as carrier and coenzyme of purity 0·2, the oxidation of phosphohexonate becomes slow when 1 mol. O_2 is absorbed and 1 mol. CO_2 has been evolved. With the Warburg & Christian system, the reaction stops when 0·6 mol. O_2 has been consumed and 0·7–0·8 mol. CO_2 has been liberated.

In view of these differences, the products formed under our conditions were investigated. The fractionation of the products followed the description given by Warburg & Christian [1937, p. 293] exactly, and details of fractionation are therefore omitted.

1·86 g. enzyme prep. A was dissolved in 130 ml. water + 28 ml. ice cold $M/50$ NaOH. 120 mg. phenazine methochloride and 9·5 mg. coenzyme II (purity 0·2) were added, and the mixture was shaken at 37·5° in O_2 with 10·0 ml. 0·09 M phosphohexonate; pH of mixture 6·4. An aliquot part of the mixture was used to measure O_2 consumption, with a control using water instead of substrate, this exp. running concurrently.

Manometric readings: 1·5 ml. enzyme solution (containing the coenzyme and phenazine methochloride) in main part of vessels. 0·1 ml. 0·09 M phosphohexonate (or water) in side bulb. 0·2 ml. KOH in inner cup. O_2. 37·5°.

Vessel no.	Add at t_0	μl. O_2 consumed after min.					
		30	60	120	240	1100	1200
1	Phosphohex.	64	100	144	195	266·5	267·5
2	Water	6	9	11	16	25·5	26·5
Difference μl. O_2: (1–2)		58	91	133	179	241	241

Calc. for 0·1 ml. $(M/10) \times 0·91$ phosphohexonate (P analysis): 204 μl. O_2 if 1 mol. phosphohexonate consumes 1 mol. O_2.

The trichloroacetic acid filtrate was worked up by Warburg & Christian's method. The filtrate contained 28·7 mg. combined P; amount of ester P added as phosphohexonate $-0.91 \times 31.0 = 28.2$ mg. Evidently little breakdown of organic P occurred. As in Warburg & Christian's exp., neutral and basic lead acetate yielded distinct precipitates of Pb salts; neutral (after removal of inorg. P): wt. 315 mg. Fractionated by Hg salt; Hg precipitable converted into Pb salt; wt. 85 mg. "A". Hg supernatant fluid reprecipitated with neutral Pb acetate: wt. 76 mg. "B". Basic Pb acetate precipitate from supernatant liquid of neutral Pb acetate precipitation, converted into Ba salt.; wt. 60 mg. "C". Salts "A" and "B" were redissolved and reprecipitated for analysis.

Analyses. (Weiler, except colorimetric P estimations on Pb but not on Ba salts. All figures are expressed on subs. dried at 40° *in vacuo* unless otherwise stated.)

Subs. "A". Found: Pb, 63·40; P, 6·26%. Warburg & Christian's Pb salt 1, prepared similarly from the Hg precipitate, had Pb, 61·5: P, 6·2%. The two were not identical however since Pb salt "A" gave the orcin test and contained more C (ratio C:P:Pb = 4·7:1·01:1·53). Consistent analyses were not obtained and the fraction was probably a mixture.

Subs. "B". Found: C, 8·92; H, 1·43; P, 5·60; Pb, 57·0%. Calc. for $C_4H_6O_8PPb_{1.5} + H_2O$: C, 8·86; H, 1·47; P, 5·71: Pb, 57·3%. Subs. "B" dried *in vacuo* at 100°. Found: C, 9·47; H, 1·56%. Calc. for $C_4H_6O_8PPb_{1.5}$: C, 9·16; H, 1·15%.

Subs. "B" thus corresponds closely with the monohydrated neutral Pb salt of a monophospho-erythronic or -threonic acid: $Pb_{\frac{3}{2}}OOC.(CH.OH).(CH.OH).CH_2O.PO_3Pb + H_2O$. In the corresponding fraction from their oxidation, Warburg & Christian [1937] obtained a Pb salt, $C_5H_8O_9PPb_{1.5}$ containing (CH_2O) in excess of our Pb salt. Taken in conjunction with the results described here, this can be taken as strong presumptive evidence that this salt of Warburg & Christian was the next higher member, namely the Pb salt of the phospho-pentonic acid. These facts, and the isolation of the pentose-containing fraction "C" described below, together provide strong experimental support for the existence of a system of progressive oxidative breakdowns of the sugar-mono-phosphoric acids, such as was previously tentatively suggested [Dickens, 1936].

It is particularly interesting that in our series, where the oxidation proceeded further, the O_2 uptake being 1 mol. per mol. substrate, instead of 0·5 mol. as in Warburg & Christian's exp., the degradation of the C-chain has proceeded one stage further. As a variety of stages of oxidation are simultaneously present, it is not possible to correlate the O_2 uptake more than roughly with the extent of breakdown. But it is noteworthy that the formation of fraction "B" in our exp. required the consumption of only half the amount of O_2 (2 mol.) calc. from the following equations:

COOH	COOH	CO₂				
CH.OH	CO	+ CHO	COOH	COOH	CO₂	COOH
CH.OH $+\frac{1}{2}O_2 =$ CH.OH	$=$ CH.OH $+\frac{1}{2}O_2 =$ CH.OH $+\frac{1}{2}O_2 =$ CO				$=$ CHO $+\frac{1}{2}O_2 =$ CH.OH	
CH.OH	CH.OH	CH.OH	CH.OH	CHOH	CH.OH	CH.OH
CH.OH	CH.OH	CH.OH	CH.OH	CH.OH	CH.OH	CH₂.O.PO₃H₂
CH₂.O.PO₃H₂	CH₂.O.PO₃H₂	CH₂.O.PO₃H₂	CH₂.O.PO₃H₂	CH₂.O.PO₃H₂	CH₂.O.PO₃H₂	

Phospho-hexonate	(?) Ba salt I (Dickens)	(?) Pb salt 1 (Warburg & Christian)	(?) Pb salt "B" (Dickens)
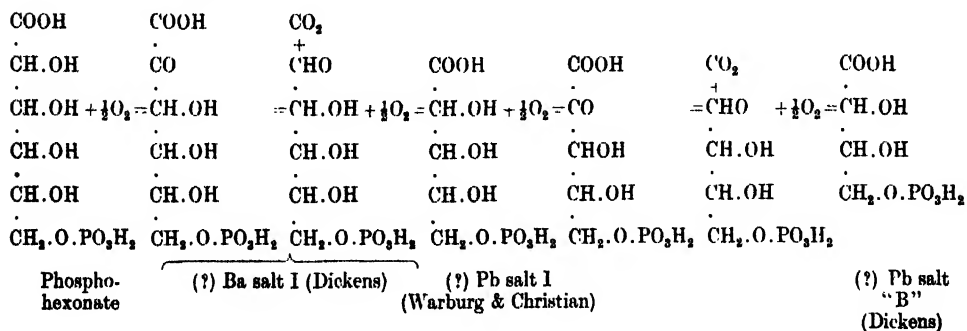
Similarly for the formation of Warburg & Christian's Pb salt 1, the consumption of 1 mol. O_2 would be required, whereas only half this amount was observed as the total O_2 uptake in their exp. (The CO_2 outputs in the two series of exp. were also about half those calc. from the above equations.) Since the calc. O_2 uptakes (and CO_2 outputs) were in excess of the observed amounts, it is evident that the excess oxidation of this stage must be accompanied by the formation of less highly oxidized products. That this was in fact the case in our exp. is shown by the analysis of Ba salt "C".

Subs. "*C*". Found: C, 23·72; H, 3·02; P, 5·21; Ba, 24·80. Calc. for $C_{11}H_{21}O_{15}PBa$: C, 23·50; H, 3·74; P, 5·52; Ba, 24·50 %. This formula corresponds empirically with the Ba salt of a compound containing 1 mol. hexose, 1 mol. pentose and 1 mol. H_3PO_4, with the addition of 1 atom O and the loss of 1 mol. H_2O. Since it gives an intense pentose reaction (see below) it is apparently a combination of 1 mol. pentose with 1 mol. phosphohexonic acid, or alternatively of 1 mol. pentose phosphoric acid with 1 mol. hexonic acid. The fate of the lost phosphoric acid mol. is unknown: since the inorganic P did not appreciably increase during the exp., either H_3PO_4 was lost during the isolation, or else was used during the incubation to phosphorylate another molecule.

Pentose reaction. The orcin-$FeCl_3$ test was used [Tollens-Elsner, 1935, p. 111]. The phosphohexonate used gave a negative reaction, or at most the faintest trace of green which developed only very slowly (2-keto-*d*-gluconate behaved similarly). Pb salt "A" and Ba salt "C" gave intensely positive reactions (*c.* 1 mg. used for test); pigment sol. in amyl alcohol with absorption bands at 595–600 and 650–60 mμ. As was to be expected from its analysis, Pb salt "B" gave no colour with the pentose test. Unfortunately the quantities were insufficient for quantitative pentose estimation; the colours in the micro-method of McCance [1926] were found to fade rapidly with pentose phosphoric acid (ribosephosphate) and to differ too much from one pentose to another.

II. OXIDATION OF PENTOSE PHOSPHORIC ACIDS

Since in the preceding part it has been shown that pentose phosphoric acids or compounds closely resembling these are formed in the oxidation of phosphohexonate by yeast enzymes, and that compounds representing still further oxidation stages are also present, particularly the substance analysing as a phosphoerythronic acid, the next step was evidently to study the oxidation of those pentose phosphoric acids that are available.

I am greatly indebted to Dr P. A. Levene, who has added so much to knowledge of pentose phosphoric acids, for generous gifts of synthetic esters, without which these experiments would have been very incomplete.

Pentose phosphoric acids

d-*Ribose-5-phosphoric acid.* (*a*) *Natural.* Ba inosinate (Laoköon, Lwów) was hydrolysed and the Ba salt isolated by the method of Levene & Jacobs [1911]. Yield 2·9 g. from 5 g. Ba salt. Recryst. from 10 ml. hot water (animal charcoal) with addition of 10 ml. alcohol to the mother liquor: yield 1·7 g. The white crystalline subs. was dried *in vacuo* over H_2SO_4 for 24 hr. Analysis (Weiler), dried at 100° *in vacuo* loss of wt. 8·64 %. Found (dry subs.): Ba, 37·7; P, 8·88 %. Calc. for $C_5H_9O_8PBa$: Ba, 37·6; P, 8·49 %: $+2H_2O$ req. 8·97 %.

(b) *Synthetic*. Dr Levene's prep. of the synthetic Ba salt [Levene & Stiller, 1934].

d-*Arabinose-5-phosphoric acid Ba salt* (synthetic). Found (Dr Levene): Ba, 37·3; P, 8·77 %. *Xylose-5-phosphoric acid (Ba salt)*. Synthetic, from Dr Levene [Levene & Raymond, 1934]. 2-*Keto*-d-*gluconic acid (Ba salt)*. Prepared according to Ohle & Koller [1924].

Preparation of c. $M/10$ *solutions of Na salts*. These were prepared freshly, as on keeping in the ice box the solutions turned yellow. 77 mg. Ba salt ground with 65 mg. Na_2SO_4 $10H_2O$ were made up to 2 ml. with addition of 0·02 ml. N HCl. Found (ribose phosphoric acid): organic P, 0·756, 0·765 $M/10$.

The same enzyme preparations, carriers and coenzymes were used as in the preceding section, unless otherwise stated, and the experimental procedure was the same.

Oxidation experiments

1. *Oxidation by Lebedew extract*. For these experiments yeast macerate, either undiluted or with $M/100$ phosphate buffer, pH 7·4, pH of mixture c. 6·4, was placed in the main part of Warburg manometers, containing in the side bulb 0·3 $M/10$ substrate (Na salts of acids), and in the inner cup 0·2 ml. KOH. O_2. 37·5°. In those exp. with extra carrier and coenzyme, 0·2 ml. $M/100$ phenazine methochloride and 0·1 mg. coenzyme II of purity 0·2 were added to the main contents.

Table VI. *Oxidation in Lebedew fluid*

Dilution with $M/100$ phosphate buffer of pH 7·4; pH of mixt. c. 6·5. 0·3 ml. $M/10$ substrates. KOH in inner cup. O_2. 37·5°.

No carrier or coenzyme added:

Source	Vol. per vessel ml.	μl. O_2 uptake in 1st hr. with substrate					
		None (water)	Glucose	Gluconic acid	2-Keto-gluconic acid	Phospho-hexonic	Ribose-5-phosph. acid
Löwenbräu:							
(Fresh)	1	40	196	116	44	212	126
(Overnight at 0)	1	40	137·5	—	—	—	163
,,	0·2	11·5	68	—	—	—	51·5

With carrier and coenzyme added:

0·2 ml. Newcastle Brewery Lebedew fluid diluted to 2·2 ml. total vol. with $M/100$ phosphate; this vol. contains 0·2 ml. $M/100$ phenazine methochloride, 0·1 mg. coenz. II of purity 0·2; 0·3 ml. $M/10$ substrate. O_2. 37·5°. KOH in inner cup.

μl. O_2 in 1st hr. with

Water	Phospho-hexonic acid	Hexose mono-phosphoric acid	Xylose-5-phosphoric acid	d-Arabinose-5-phosphoric acid	d-Ribose-5-phosphoric acid	2-Keto-gluconic acid
42·5	186	—	—	46·5	146	—
29	—	205	27	34	108 (synthetic) 92 (natural)	32

Table VI shows that ribosephosphate is oxidized with both specimens of Lebedew fluid, whether diluted or undiluted, with or without the addition of the extra carrier and coenzyme. Lebedew fluid kept overnight in the ice box still oxidizes vigorously. Natural and synthetic ribosephosphates are both oxidized, but xylosephosphate and arabinosephosphate are little or not at all affected when phenazine methochloride is the main carrier present. The figures for keto-gluconic acid are included because of the suggestion that phosphoketogluconic

acid may be an intermediate in hexose oxidation. The unphosphorylated compound was not appreciably oxidized. Figures for phosphohexonate and hexosemonophosphate are included for comparison.

Fig. 6 shows the course of the reaction and the comparison between natural and synthetic ribose esters.
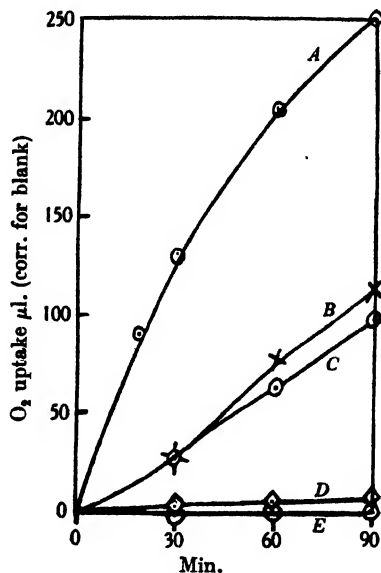
Fig. 6. Oxidation by Lebedew fluid (Newcastle Breweries). 0·3 ml. M/10 substrate +0·2 ml. Lebedew fluid + phenazine methochloride +0·1 mg. coenzyme to 2·2 ml. with M/100 phosphate. O_2. 37·5°. A: hexosephosphate. B: ribosephosphate (synthetic). C: ribosephosphate (natural). D: 2-ketogluconic and d-arabinose phosphate. E: d-gluconic acid.

2. *Boiled enzyme.* Table VII shows that the activity of Lebedew fluid towards ribosemonophosphate is destroyed by boiling.

Table VII. *Boiled and unboiled Lebedew fluid*

0·3 ml. Löwenbräu Lebedew fluid diluted to 1·5 ml. with M/30 phosphate of pH 7·4; pH of mixt. c. 6·5. 1·5 ml. per vessel either untreated or placed in a boiling water bath for 10 min. 0·3 ml. M/10 ribose phosphate or 0·3 ml. water. O_2. 37·5°.

μl. O_2 uptake in 1st hr.

	Ribosephosphate		Water	
Enzyme ...	Unboiled	Boiled	Unboiled	Boiled
	68·5	16	23	16·5

3. *Acetate precipitate from Lebedew fluid.* The enzyme used in the previous section (prep. A) is not suitable for the study of the oxidation of pentose phosphoric acids, since its activity is low and variable with this system at any rate when phenazine methochloride is used as carrier (Table VIII). That the preparation used was fully active towards hexosemonophosphate or phosphohexonate is shown by the control exp. with these substrates. Evidently different systems are concerned in the oxidation of pentose and hexose phosphoric acids. This explains the fact that the pentose esters were only in part further oxidized in our previous experiments with phosphohexonate.

Table VIII. *Acetate precipitate A*

20 mg. enzyme dissolved in 1 ml. $M/30$ phosphate buffer of pH 7·4; pH 6·4: 0·2 ml. $M/100$ phenazine methochloride; 0·1 mg. coenzyme I of purity 0·2. 0·2 or 0·4 ml. $M/10$ substrate. O_2. 37·5°. 0·2 KOH in inner cup.

| | | | Ribose-5-phosph. | | Arabinose-5-phosph. (synthetic) | | Hexose-mono-phosph. | Phospho-hexonate |
	Min.	Water	Synthetic 0·2 ml.	Natural 0·2 ml.	0·2 ml.	0·4 ml.	0·2 ml.	0·2 ml.
2 months' old enzyme	60	4·5	7·5	13·5	—	—	147·5	—
Freshly prepared enzyme	60	0·5	—	36·5	5	4·5	—	135
	120	1·0	—	64	12·5	18	—	214
	180	1·5	—	77	16	23	—	256

4. *Dialysed Lebedew fluid.* Lebedew fluid was dialysed for various times in a "Visking" cellophane tube against running tap water in a shaking machine. The results are shown in Table IX.

Table IX. *Dialysed Lebedew fluid*

1 ml. dialysed Lebedew fluid; 0·1 mg. coenzyme II of purity 0·2, or 0·2 mg. purified cozymase; 0·3 $M/10$ ribose-5-phosphoric acid or 0·3 ml. water. O_2. 37·5 . 0·2 ml. KOH in inner cup.

μl. O_2 uptake

| Dialysis hr. | Exp. min. | Water | | | Ribose phosphoric acid | | | |
		Water	Coenz. I	Coenz. II	Water	Coenz. I	Coenz. II	Coenz. I + II
4	60	20·5	—	25	110	—	146	—
16	60	0·5	—	2	6	—	38	—
	100	1	—	2	10	—	62·5	—
16	60	—	0	—	1	4·5	53	50
	120	—	0	—	5	10	109	116

4 hr. dialysis do not sufficiently remove the coenzymes, although an increase is now detectable on addition of coenzyme II (purity 0·2 throughout this work). On the other hand after 16 hr. the oxidation of ribosephosphate is very small unless coenzyme II is added, when it is about the same as in the original Lebedew fluid (Table I) after subtraction of the corresponding blanks without substrate. The blank is now very small in the dialysed Lebedew fluid. Cozymase (tested for activity with the lactic dehydrogenase) caused only a trifling increase, which may well be due to a trace of coenzyme II still contained in the purified cozymase. Addition of cozymase and coenzyme II together did not give any appreciable increase over coenzyme II alone. (Note however that the coenzyme II employed in these experiments certainly contained some cozymase.)

5. *Dried enzyme from dialysed Lebedew fluid. Preparation.* 150 g. Löwenbräu, Munich, dried yeast was macerated with 450 ml. distilled water for $2\frac{1}{2}$ hr. at 35°. The centrifuged Lebedew fluid (190 ml.) was dialysed as described above for 16 hr. against running tap water with shaking. After centrifuging from a considerable deposit, the supernatant, almost clear liquid was evaporated over H_2SO_4 in flat dishes in large vacuum desiccators evacuated with a Hyvac pump until the contents froze. The residue became almost dry in a few hr. The dry, light coloured, feathery powder weighed 6·2 g. For use, 80 mg. were taken in 1 ml. (equiv. 2 ml. original Lebedew fluid) distilled water per vessel. No carrier was added in these experiments, only substrate and 0·2 mg. coenzyme II being added. The solution

was centrifuged from a little denatured protein before use. The preparation retains it activity for long periods if kept cold and dry.

Activity of the dried preparation. Fig. 7 shows the activity with ribose-phosphate, and that in the absence of coenzyme no significant O_2 uptake occurs. Since the O_2 consumed in Fig. 7 (0·1 ml. 0·076 $M = 167 \mu l$. ribose) exceeds
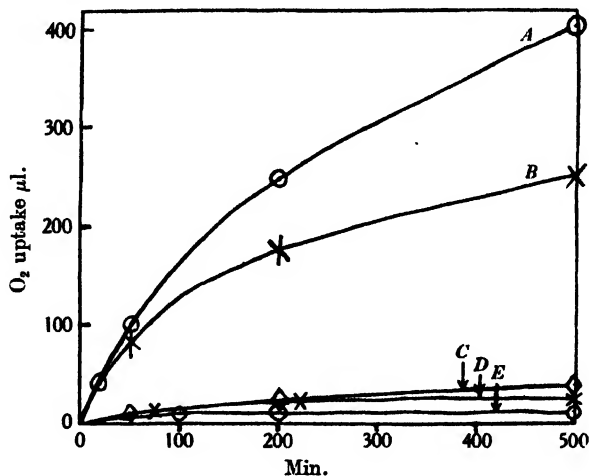
Fig. 7. *A*: 0·3 ml. 0·076 *M* ribosephosphate. *B*: 0·1 ml. ribosephosphate. *C*: 0·3 ml. 0·076 *M* ribosephosphate, no coenzyme. *D*: no substrate, with coenzyme. *E*: no substrate, no coenzyme.
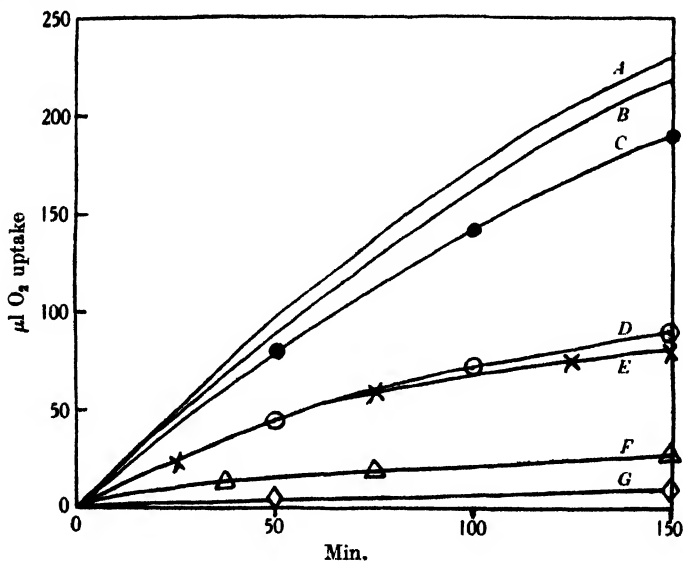
Fig. 8. Oxidation of various substrates (0·3 ml. *M*/10) by dried dialysed Lebedew fluid. *A*: phospho-hexonic. *B*: hexosemonophosphate. *C*: ribosephosph. (Synthetic). *D*: *d*-arabinose phosph. *E*: xylosephosph. *F*: blank without substrate, with coenz. *G*: blank: no coenz., no substrate.

1 mol. O_2 per mol. ribosephosphate, it is evident that the oxidation of this compound proceeds beyond the phosphoribonic acid, and even beyond the ketoribonic acid, if this is indeed formed.

Fig. 8 shows the action on various substrates. With this preparation, unlike the system consisting of Lebedew fluid with phenazine methochloride as carrier (Table VI), xylosephosphate and arabinosephosphate are also oxidized, but at less than half the rate of the ribose ester, which in this experiment was also the synthetic compound. It is interesting to note that this is oxidized almost as rapidly as the two hexose phosphoric esters. The action on the arabinose and xylose compounds which did not occur with phenazine methochloride but occurs with the natural carrier may be compared with the different behaviours of Neuberg and Robison esters with phenazine methochloride and flavin enzyme already described [Dickens & McIlwain, 1938].

6. *CO_2 formation.* Fig. 9 shows that CO_2 formation and O_2 consumption are nearly equimolecular.
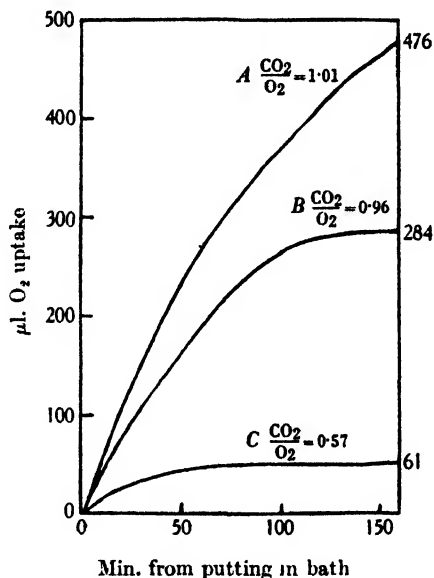
Fig. 9. CO_2 formation and O_2 uptake with dried dialysed Lebedew fluid + 0·2 mg. coenzyme. *A*: 0·3 $M/10$ ribosephosph. *B*: 0·1 $M/10$ ribosephosph. *C*: no added substrate.

For this exp. R.Q. vessels [Dickens & Šimer, 1930] were used with enzyme, coenzyme and substrate in the inner part, $Ba(OH)_2$ as CO_2 absorbent, and HCN in the side bulb. The readings were extrapolated for the 1st 10 min. of temperature equalization. During this exp. inorganic P increased by about 0·4–0·5 of the equivalent of the O_2 uptake.

7. *Oxidation by laked blood cells.* Each vessel contained the cells from 2 ml. horse blood, washed three times with 0·9 % NaCl, and diluted to 2 ml. with water. 0·3 ml. $M/10$ substrate was added to each, and 0·1 mg. methylene blue dissolved in 0·2 ml. water was tipped in from the side bulb when equilibrium was reached. 0·2 ml. KOH in inner cup. Fig. 10 shows the O_2 uptakes corrected for the O_2 evolution in the blank [cf. Warburg & Christian, 1931]. Of the three pentose phosphoric acids, the ribose compound is the only one to be attacked. Curves for hexosemonophosphate and phosphohexonate are included for comparison.

8. *Oxidation in tissue slices.* In intact cells diffusion problems probably prevent the entry of phosphorylated products. On the other hand, pentoses are not apparently readily phosphorylated within the cell, though our evidence shows

that they may arise from phosphorylated hexoses which the cell is able easily to manufacture. Consequently, in intact cells oxidation of added pentoses is hardly to be expected, and in fact did not occur (Fig. 11).
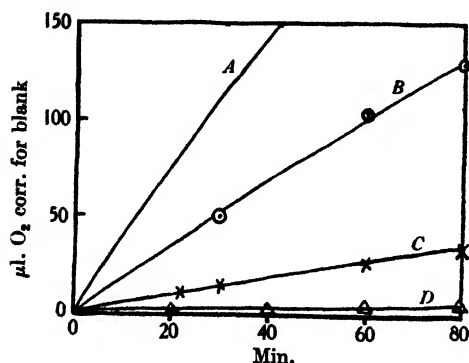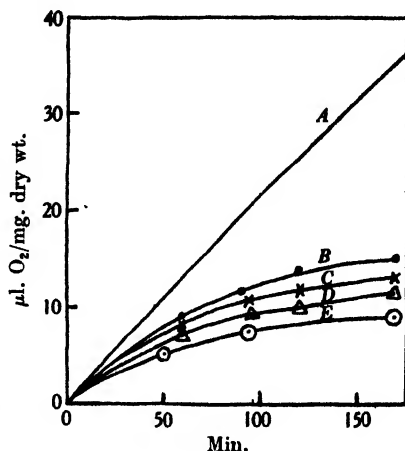
Fig. 10.   Fig. 11.

Fig. 10. Oxidation by laked horse blood. A: hexosemonophosph. B: phosphohexonic. C: ribose-phosph. D: arabinosephosph. *and* xylosephosph.

Fig. 11. Oxidation in brain slices of rat. $M/90$ substrates in phosphate-Ringer solution. O_2. 37·5°. A: glucose. B: d-ribose. C: l-arabinose. D: d-arabinose and without added substrate. E: i-erythritol.

Although not entirely relevant here, it may be mentioned that kidney slices can readily oxidize 2-keto-d-gluconic acid, as the following exp. shows.

Rat kidney slices. Phosphate-Ringer soln. O_2. 37·5°. 0·2 ml. KOH in inner cup.

	Q_{O_2}		
	1st	2nd	3rd hr.
Control	− 15·1	− 10·9	− 9·6
Na 2-ketogluconate $M/50$	− 19·0	− 16·7	− 15·6

DISCUSSION

It is proposed to discuss these results in detail when a sufficient quantity of the oxidation products has been prepared for their identification. At present the results, particularly the fact that 5-C and 4-C compounds have been identified among the products, provide support for the scheme of progressive oxidation and decarboxylation previously advanced [Dickens, 1936].

The fact that d-ribose-5-phosphoric acid, and not the expected d-arabinose-5-phosphoric acid, should prove to be the physiologically oxidizable (and fermentable [Dickens, 1938]) substrate introduces a new factor into the problem. Evidently if d-ribose is to arise from d-glucose by removal of the terminal group, an epimerization must occur. It is recognized that inversion may occur in an asymmetrically disposed C atom with an attached H atom and an adjacent =CO group. A type of Walden inversion could theoretically occur either as a result of decarboxylation or by the introduction and subsequent removal of a second phosphoric acid group. These possibilities will be discussed when more direct evidence of this inversion has been sought.

SUMMARY

I. *Oxidation of phosphohexonate*

An enzyme has been prepared from Lebedew maceration fluid by precipitation at pH 4·6, washing with acid and drying. It has been used to study the oxidation of phosphohexonic acid. There is an optimum activity between pH 6·3–7·5. The action is inhibited by phosphate like the hexosemonophosphate system, but less powerfully. With pure coenzyme II oxidation of phosphohexonic acid proceeds until $\frac{1}{2}O_2$ per mol. is consumed. At this stage the product, prepared as a Ba salt, appears to consist of a mixture of phosphoketohexonate and phosphopentonic acid, as judged by its analysis and mode of preparation.

When less pure coenzyme is used the reaction proceeds further, the end-products and end-point being altered. The O_2 uptake and CO_2 output are now each about 1 mol. per mol. substrate, an amount different from that seen in experiments of Warburg & Christian [1937] with a different preparation of enzyme. Among the end-products an analytically pure compound corresponding to a phosphorylated 4-C monocarboxylic dihydroxy-acid—possibly phospho-erythronic acid—was isolated. Two other fractions gave a definite reaction for pentose by Bial's test.

II. *Oxidation of pentose phosphoric acids*

Lebedew maceration fluid from dried brewers' yeast oxidizes d-ribose-5-phosphoric acid vigorously, and to a less extent d-arabinose- and xylose-5-phosphoric acids. When phenazine methochloride is the carrier the latter two substrates are not appreciably oxidized. The activity is destroyed by boiling. The acetate precipitate from Lebedew fluid is feeble towards pentosephosphates, at least with phenazine methochloride as carrier. An active dialysed preparation was made from Lebedew fluid; it can be kept as dry powder and is inactive without the addition of coenzyme II. With coenzyme II oxidation is vigorous with ribosephosphate, only about half as fast with the other pentosephosphates. CO_2 evolution and O_2 absorption are about equal to 1 mol. of each per mol. pentosephosphate. Laked horse blood oxidizes ribosephosphate slightly in presence of methylene blue; the other two pentose esters are not attacked.

Intact cells being impermeable to phosphoric sugar esters do not oxidize them, nor are free pentoses oxidized by brain slices. Kidney slices readily oxidize 2-keto-d-gluconic acid.

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CCXII. YEAST FERMENTATION OF PENTOSE PHOSPHORIC ACIDS

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In all references to pentoses in textbooks on sugars, the fact that they are not fermented by yeast [Salkowski, 1900] is prominent. In the following it is shown that while the pentoses are not fermented appreciably either by yeast cells or by Lebedew extract, phosphorylated pentoses are fermented, and that in the case of the d-ribose-5-phosphoric ester, the fermentation by yeast extract is about as rapid as that of glucose or hexosemonophosphate. The fermentation of d-ribose-5-phosphoric acid requires the presence of cozymase, yeast enzyme and inorganic phosphate, and probably of metal salts (Mg and Mn) also. (Adenylic acid was present in the cozymase used.) The products of fermentation are per mol. ribosephosphate, 1 mol. CO_2, 1 mol. alcohol and 1 mol. inorganic phosphate. In addition an unknown, HIO_4-reducing substance appears, which may perhaps be glycol.

The natural pentose phosphoric acid of animal and yeast cells—d-ribose-5-phosphoric acid—is fermented far more rapidly than either d-arabinose-5-phosphoric acid or the corresponding xylose compound. This makes it possible that d-ribose-5-phosphoric acid may play an important part as an intermediate both in aerobic fermentation and in sugar oxidation [Dickens, 1938]. These facts may eventually prove of significance in connexion with the mechanism of the Pasteur reaction and in carbohydrate metabolism generally. The part played by pentose phosphoric acids in metabolism is being further studied.

Methods

Alcohol estimation. The method of Friedemann & Klass [1936] was used and proved satisfactory provided that all the precautions described were rigidly observed. Erratic results are obtained unless all glassware is cleaned with chromic-sulphuric acid immediately before use. The distillation apparatus was modified to suit existing equipment, and with the glass condenser used the recovery was as follows: μl. alc. taken (1 mM. $= 22400 \mu$l.); 224, 224, 45, 45; recovered; 90, 91, 93·5, 88% mean recovery 90·5%. Redistillation from $Ca(OH)_2$-$HgSO_4$ was always used after the distillation from $HgSO_4$, to ensure removal of interfering substances. Pentose phosphoric esters did not give any volatile titratable material. It is essential to have a large excess of $KMnO_4$ during oxidation.

Reducing substances. The Cu reduction method of Somogyi [1937] was adapted for the separate estimation of pentose and pentose phosphoric acid. The reducing values of d-ribose, d-ribose-5-phosphoric acid and glucose found for our preparations were respectively 58%, 57% and 100% for equimolecular quantities (ribose-phosph. calc. on P content of soln. of Na salt). Neutral Pb acetate was found to remove ribosephosphate practically quantitatively from its solution and the loss of free ribose under the same conditions was negligible.

Method. The proteins are removed with tungstic acid as usual. The perfectly clear centrifugate is pipetted into a dry centrifuge tube, an aliquot being used for total reducing substance. The ribosephosphate is precipitated by neutral Pb acetate, allowing to stand for $\frac{1}{4}$ hr. Centrifuging removes the lead salt and the clear or faintly opalescent supernatant fluid is pipetted into a dry centrifuge tube, excess $M/2$ Na_2HPO_4 added, Pb phosphate removed by centrifuging and a measured amount of the perfectly clear supernatant fluid used for the estimation of reducing substance not precipitable by neutral Pb acetate. 0·03 ml. $M/10$ solution of ribosephosphate per ml. tungstate filtrate was the concentration usually employed for the Pb precipitation.

HIO_4 titration. This was used for detection and estimation of substances containing two or more adjacent free OH groups (micro-method modified from Fleury & Fatome [1935]). It was found that 1 ml. $M/50$ soln. of each of the following required the approximate amounts of $N/50$ I_2 stated: glycerol, 4·0; glucose, 8·8; ribose, 6·4; hexosemonophosphate, 7·1; ribosemonophosphate, 5·3; glycol, 2·0 ml. The method is applicable to the Na_2HPO_4 supernatant fluid mentioned above, provided that it is acidified to litmus before adding the HIO_4. Quantities below 1 ml. $M/100$ can be estimated with accuracy, but the method is only applicable if sugars and interfering substances are known to be absent.

Materials

Pentose-5-phosphoric acids. The natural *d*-ribose-5-phosphoric acid and synthetic *d*-arabinose- and xylose-5-phosphoric acids (Levene) used have already been described [Dickens, 1938], together with the prep. of the $M/10$ soln. of the Na salts. *d-Ribose* was made from guanosine (B.D.H.) according to Levene & Clark [1921]. It crystallized readily in the desiccator.

Cozymase was a purified prep. made in this laboratory by Dr Weil-Malherbe.

Muscle adenylic acid was obtained from Fraenkel and Landau. It appeared to contain cozymase, and as the cozymase contained adenylic acid, adenylic acid was not added in more than a few exp.

Mg-Mn soln. 0·025 M $MgSO_4$ containing 0·005 M $MnSO_4$.

Ca hexosediphosphate B.D.H. prep. *Mg hexosediphosphate* kindly presented by Prof. Schoeller, Schering-Kahlbaum. $M/50$ soln. was used, the Ca salt being dissolved by partial decomp. by Na_2SO_4.

Glycollaldehyde was prepared from dihydroxymaleic acid by distillation after heating with pyridine [Fischer & Taube, 1927]: it crystallized readily after distillation.

Enzyme preparations. Lebedew fluid was prepared from Löwenbräu, Munich, bottom yeast. The dialysed, dried preparation already described [Dickens, 1938] was also made from this yeast.

Fermentation of pentose and pentosephosphoric acids by
Lebedew fluid and by yeast cells

Yeast cells. D.C.L. Company's yeast was washed three times at the centrifuge with dist. water and a 2% suspension in $M/25$ KH_2PO_4 prepared. 1 ml. per vessel was transferred to Warburg manometers and the substrates were added to make the conc. $M/15$. Fermentation was measured in N_2 at 37·5° (Fig. 1). Ribose was not at all fermented, ribosephosph. was slightly attacked, but the action was very small compared with the very vigorous fermentation of glucose and may have been due to a trace of impurity, but in such cases there is usually a sharp fall of reaction rate, which was not so here.

Lebedew fluid. Fig. 2 shows that contrary to what happens with the intact cells, fermentation with ribose phosph. is very vigorous in Lebedew extract, and ribose is also attacked, though relatively very much more slowly.

Products and end-point of fermentation of ribosephosphate by Lebedew fluid. Fig. 3 shows the course of the reaction. The end-point of fermentation of 9 ml.

Lebedew fluid with 2·4 ml. 0·76 $M/10$ ribosephosph. was 7840 μl. CO_2; blank (9 ml. + 2·4 ml. water) 735 μl. Diff. 7105 μl. CO_2. Ribose P taken = 8170 μl. (22400 μl. = 1 ml. M soln.). Ratio: mol. CO_2/mol. ribose P = 0·86.

Fig. 1.

Fig. 2.

Fig. 1. Yeast cells. Fermentation of glucose, ribose and ribosephosphate. Conc. $M/15$.

Fig. 2. Fermentation with Lebedew macerate (1 ml.). Conc. of substrates in mixture are shown.
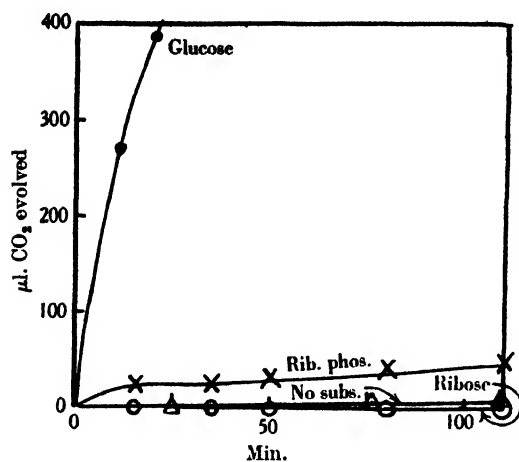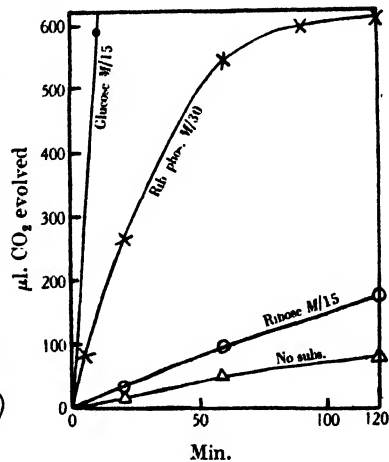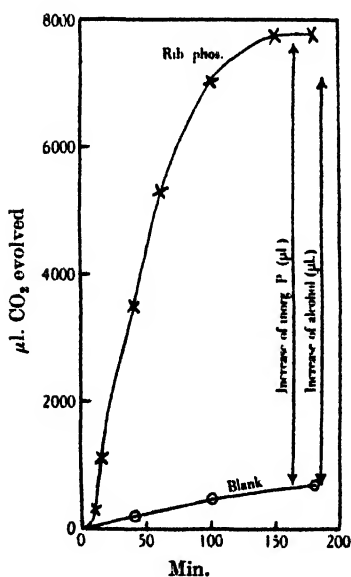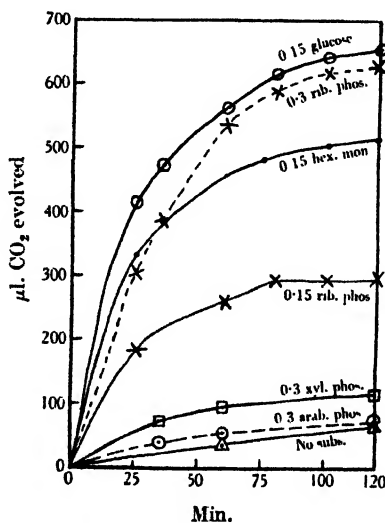
Fig. 3.

Fig. 4.

Fig. 3. Fermentation of ribosephosphate by Lebedew fluid: end-point, alcohol and P estimations.

Fig. 4. Fermentation of various substrates by Lebedew fluid (1 ml.): ml. $M/10$ substrates added as shown.

Increase of inorganic P in trichloroacetic acid filtrate $= 6950\,\mu$l. $= 98\%$ of CO_2 evolved and $= 85\%$ of ester P present in the ribosephosph. Alcohol increase, $6300\,\mu$l. $= 90\%$ of CO_2 formed (1 m.Mol. H_3PO_4 or $C_2H_5OH = 22,400\,\mu$l.)

This exp. shows that ribosephosph. is decomposed to the extent of 86% by Lebedew fluid; that CO_2 equivalent to 85% of 1 mol. CO_2 per mol. total ribose-phosph. is liberated; that the alcohol formed, assumed to be ethyl alcohol, is 77% of the total ribose ester taken, and that inorganic phosphate is split off equivalent to the CO_2 formed.

Fermentation of other pentose phosphoric acids by Lebedew fluid

Fig. 4 shows the result of incubating various substrates with Lebedew fluid. Arabinosephosph. and xylosephosph. are only slightly fermented. With ribose-phosph. we have seen that only 1 mol. CO_2 is liberated per mol. substrate, hence to compare the rate of reaction with those of hexosemonophosphate and glucose, which give 2 mol. CO_2 per mol., half quantities of these substrates were used (Fig. 4). When this is done, the rate of fermentation of ribosephosph. is close to that of glucose, and about equal to that of hexosemonophosphate (Robison ester).

In view of the fact that the pentoses themselves are practically non-ferment-able, this observation opens a new field for investigation.

The alcohol estimations in the exp. of Fig. 4 were as follows:

Table I. *Alcohol estimations and CO_2 liberation*

Substrate	Water	0·3 ml. 0·05 M glucose	0·15 ml. 0·091 M hex. mono-phosph.	0·3 ml. 0·076 M ribose phosph.	0·3 ml. 0·08 M arab. phosph.	0·3 ml. 0·08 M xyl. phosph.	0·15 ml. 0·076 M rib. phosph.
μl. substrate	0	336	303	511	538	538	256
μl. CO_2 evolved	64	656	514	625	75	115	298
Increase CO_2 over blank	0	592	450	561	11	51	234
Calc. CO_2 for mols.	—	2	2	1	1	1	1
„ μl.	—	672	606	511	538	538	256
$\left(\dfrac{\text{Found}}{\text{Calc.}}\right) CO_2 \%$	—	88	74	110	2	9·5	91
μl. alcohol (increase over blank)	—	732	—	582	—	—	—

With Lebedew fluid, the alcohol increase, being small in proportion to the relatively high alcohol content of the macerate, can only be measured approximately. The CO_2 retention of Lebedew fluid, despite its acid reaction, is also appreciable. These objections apply less to the enzyme experiments.

Fermentation of ribosephosphate by dried enzyme

Each Warburg vessel contained 80 mg. dried dialysed Lebedew extract dissolved in 1 ml. water and centrifuged. 0·1 ml. Mg-Mn soln., $M/2$ KH_2PO_4, $M/50$ hexose-diphosph., adenylic acid (1 mg./ml.), and cozymase (2 mg./ml.), and 0·2 ml. acetaldehyde (25 mg./ml.) and glycollaldehyde (25 mg./ml.) were added as required. In all exp. unless otherwise stated 0·3 ml. $M/10$ ribosephosphate and 0·3 or 0·15 ml. $M/10$ hexosemonophosphate were the substrates used. N_2. $37·5°$.

The results are shown in Figs. 5–7. ·

Induction period. Fig. 5 shows that fermentation with the dried enzyme sets in only after the addition of an activator (in one exp. a spontaneous onset of fermentation after 30 min. was observed; this may have been due to accidental

contamination with some activating substance). In the exp. of Fig. 5 the actions of acetaldehyde with ribosephosphate and hexosemonophosphate are compared.
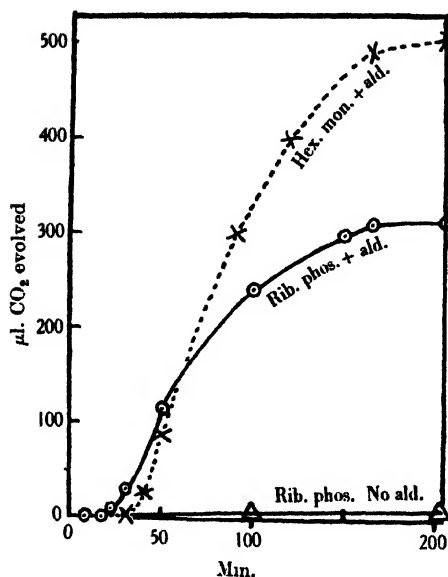
Fig. 5.
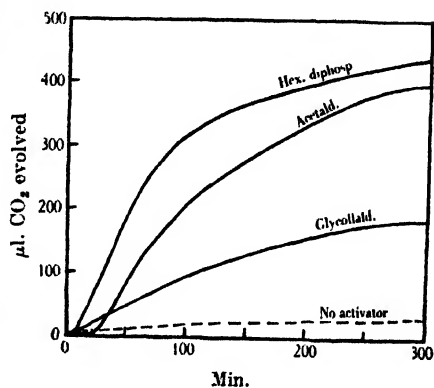
Fig. 6.

Fig. 5. Dried enzyme: activation of fermentation of ribosephosph. by acetaldehyde.

Fig. 6. Dried enzyme: comparison of activators (acetald. $M/20$, glycollaldehyde $M/30$, hexose-diphosphate $M/900$).
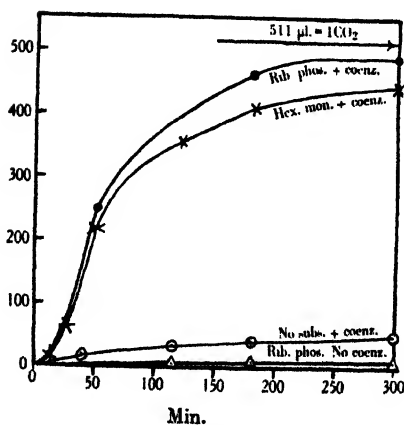
Fig. 7.
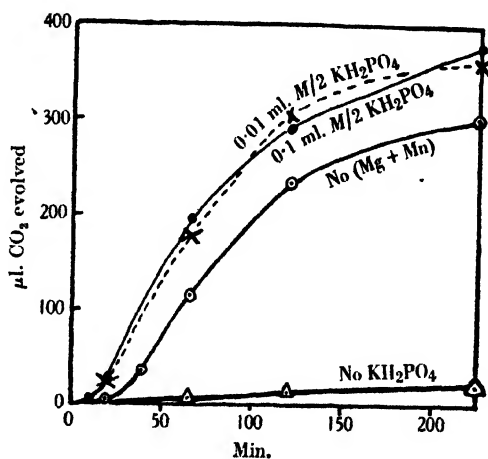
Fig. 8.

Fig. 7. Dried enzyme: need for coenzyme I. 0·3 ml. ribosephosph., 0·15 ml. hexosemonophosph.

Fig. 8. Dried enzyme: need for inorg. P; effect of addition of Mg and Mn.

Fig. 6 gives a comparison of the activation by acetaldehyde with that of hexosediphosphate and glycollaldehyde. Glycollaldehyde was tested because it has often been suggested as a breakdown product of pentoses and was described by Neuberg & Sandberg [1921] as an exceptionally vigorous activator in hexose fermentation. It was less efficient than the other two substances, of which hexosediphosphate was the better. (0·1 ml. M/50 hex. diphosph.=45 μl. per vessel.) The action resembles that well known in hexose fermentation. This amount of hexosediphosphate was added in all the following experiments.

Coenzyme. Fig. 7 shows that cozymase is necessary for fermentation to proceed, and also shows the end-point of the fermentation clearly. Many similar results were obtained. The cozymase used was that tested previously [Dickens, 1938] and shown to be free from coenzyme II; it probably contained adenylic acid.

Phosphate and mineral salts. Fig. 8 shows that inorganic phosphates are a necessary component for fermentation to occur. (Dialysed enzyme free from inorg. P; other reagents also, except ribosephosph. which contained 0·06 mg. (40 μl.) inorg. P in the 0·3 ml. used.) Addition of 112 μl. inorg. P produced full activation, smaller quantities were not tested. By analogy with hexose fermentation this suggests the primary formation of a pentosediphosphate; evidence for which is being sought.

It is also clear from Fig. 8 that the reaction is influenced by the presence of Mg^{++} and Mn^{++}. (0·1 ml. Mn-Mg soln. added contained 55 μl. Mg and 11 μl. Mn.) Special precautions would be necessary to obtain an enzyme prep. free from traces of these metals.

The fermentation of pentose phosphoric acid was in all these respects like that of hexosemonophosphate, which has been so much studied.

Alcohol estimations

Table II. *Alcohol estimations with dried enzyme*

Substrate	Coenzyme	Activator	Time of exp. (min.)	Alcohol μl.	CO_2 evolved μl.
Ribosephosph.	None	None	150	11	0
			240	0	0
,,	+	None	240	69	32
,,	+	Acetald.	240	475	397
,,	None	Hexosediphosph.	240	26	11
,,	+	,,	240	369	439
Water	+	,,	240	38·5	66·5
Hexosemonophosph.	None	,,	310	5·5	1·5
,,	+	,,	310	624	682

The results show that the CO_2 output is on the whole in reasonably good agreement with the alcohol formation, on the basis of the assumption that 1 ml. pentose ester gives 1 mol. CO_2 and 1 mol. alcohol. A great many experiments and long practice with the micro-estimation would be necessary to prove the exact degree of agreement.

In view of the fact that in the one exp. with acetaldehyde 1100 μl. of this substance were added, the degree of its removal may be regarded as fairly satisfactory, and makes it very improbable that volatile aldehydes formed in the fermentation could interfere seriously with the alcohol estimation.

The exp. of Table III shows that nearly all the ester P added is split off during the incubation, whether fermentation occurs or not (with or without cozymase).

Table III. *Phosphate balance sheet*

Vessel no. ...	1	2	3	4	5
Contents of vessel:					
A. Enzyme + KH_2PO_4 + Mg + Mn	+	+	+	+	+
B. Hexosediphosph. 0·1 ml. $M/50$	+	+	+	+	+
C. Cozymase, 0·2 mg.	−	+	+	−	+
Substrate:					
D. Ribosemonophosph. 0·3 ml.	+	+	−	−	−
E. Hexosemonophosph. 0·15 ml.	−	−	−	+	+

310 min. incubation. N_2. 37·5°

Inorganic P mg. added in component:					
A	1·37	1·37	1·37	1·37	1·37
B	0·00	0·00	0·00	0·00	0·00
C	0·00	0·00	0·00	0·00	0·00
D	0·06	0·06	0·00	0·00	0·00
E	0·00	0·00	0·00	0·03	0·03
Total at start	1·43	1·43	1·37	1·40	1·40
Found at end	2·16	2·21	1·53	1·81	1·87
Increase inorg. P	**0·73**	**0·78**	**0·16**	**0·41**	**0·47**

Organic P (acid hydrolysable, not precip. trichloroacetic acid) mg. added in component:					
A	0·00	0·00	0·00	0·00	0·00
B	0·13	0·13	0·13	0·13	0·13
C	0·00	0·01	0·01	0·00	0·01
D	0·71	0·71	0·00	0·00	0·00
E	0·00	0·00	0·00	0·38	0·38
Total at start	**0·84**	**0·85**	**0·14**	**0·51**	**0·52**
$\dfrac{\text{Increase inorganic P} \times 100}{\text{Total organic P added}}$ %	87	92	110	80	90

Loss of reducing substance on fermentation

Table IV shows the balance sheet of an experiment in which the increase of inorganic P is correlated with the appearance of a non-precipitable[1] reducing

Table IV. *Incubation of ribosephosphate with and without coenzyme*

	No coenzyme		+Coenzyme	
	1	2	3	4
Vessel no.	Preformed	Final	Preformed	Final
Incubation time, min.	0	330	0	330
Inorg. P, μl. (31 mg. = 22,400 μl.)	1530	2370	1530	2400
Ribosephosph. ester-P added, μl.	1020	1020	1020	1020
	Increase on		Increase on	
Increase inorg. P, μl.	no. 1	840	no. 3	870
CO_2 evolved, μl.	—	11·5	—	845
Total Cu-reducing subs. per vessel, calc. as ribose (or ribosephosph.), μl.	1031	1386	1031	342
Change during exp., μl.	+337		−689	
Non-precipitable Cu-reducing subs., μl.	144	922	144	160
Change during exp., μl.	+778		+16	
Orcin-FeCl$_3$ test on Pb acetate supernatant liquid	Trace	+ + +	Trace	Negative
Periodate titration on Pb acetate supernatant liquid: I$_2$ equiv. of HIO$_4$ reduced; ml. $N/100$ I$_2$	8·8	30·2	8·4	18·7
Increase during exp.	21·4		10·3	
Calc. I$_2$ equiv. of reducing substance liberated (assumed to be ribose)*	22·6		0·5	

* Taking 1 ml. $M/100$ ribose (= 224 μl.) = 6·5 ml. $N/100$ I$_2$.

[1] Using the term to mean not precipitated by neutral Pb acetate.

substance, presumably ribose, in the absence of coenzyme, and with the virtually complete disappearance of reducing substance in excess of that of the blank, when ribosephosphate is incubated in the presence of cozymase.

The results of Table IV show that pentose, as indicated by the orcin-FeCl₃ test, completely disappears from the vessel containing ribosephosphate and coenzyme after fermentation is complete (ribosephosph. gives the test as strongly as free ribose). There is an increase in the HIO_4 titration on the Pb non-precipitable fraction which does not correspond with the small change of reducing value of the same fraction. It suggests the formation of a new product during fermentation, and from the fact that this substance reduces HIO_4 it probably contains two adjacent OH groups.

Nature of the HIO_4-reducing substance. It has been shown above that the fermentation of 1 mol. *d*-ribose-5-phosphoric acid results in the formation of 1 mol. CO_2, 1 mol. alcohol and 1 mol. H_3PO_4. The residue of the 5-C chain remains to be accounted for:

$$C_5H_9O_5.PO_3H_2 + H_2O = C_2H_5.OH + H_3PO_4 + CO_2 + C_2H_4O_2$$
$$\text{\textit{d}-ribose-5-phosp.} \qquad \text{ethyl alc.} \qquad\qquad \text{Unknown 2-C}$$
$$\text{product}$$
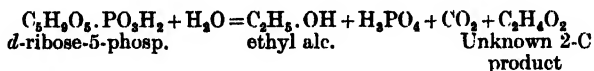
The most obvious choice would be glycollaldehyde, which arises during the action of Na_2CO_3 on pentoses [Fischler & Boettner, 1928] together with methylglyoxal, the latter possibly from intermediate formation of triose. Glycollaldehyde has also been suggested as an intermediate in the fermentation of pentose by the pentose-fermenting bacteria by Peterson & coworkers and by van der Lek [lit. quoted Kluyver, 1935]. Titration of the $NaHSO_3$-combining substances and distillation of volatile acids revealed no significant increase, however; hence neither glycollaldehyde nor acetic acid appears as a main product. It should be possible by fixation experiments to obtain evidence of the intermediate formation of glycollaldehyde, if this occurs. This is now being tested.

Neuberg & Schwenk [1916] found that glycollaldehyde was reduced by vigorously fermenting yeast to form glycol. The following exp. however suggests that glycollaldehyde, if formed, would probably undergo dismutation under our conditions.

200 mg. dialysed dried Lebedew prep. were dissolved in 2 ml. water +1 ml. 1·3% $NaHCO_3$. 0·4 mg. cozymase was added and the soln. (1 ml.) placed in the main part of three Warburg vessels. $N_2 + CO_2$, 37·5°. The side bulbs contained 5 mg. glycollaldehyde, 5 mg. acetaldehyde and water respectively. A steady positive pressure developed only in the vessel with glycollaldehyde. It amounted to the equiv. of 100 μl. CO_2 per hr.: water control 12 μl. CO_2 per hr.

The nature of the products formed is being investigated, but it is probable that dismutation to glycollic acid and glycol had occurred, with displacement of CO_2 from the bicarbonate.

This result shows that the formation of glycol ($\frac{1}{2}$ mol.) and glycollic acid ($\frac{1}{2}$ mol.) from glycollaldehyde (1 mol.) could occur with our enzyme prep. As yet however tests for glycollic acid [Denigès, 1909] have not been successful. On the other hand, there is an increase in the HIO_4-titratable material after fermentation, which might indicate the presence of glycol. The substance is non-Cu-reducing and is not precipitable by neutral Pb acetate and the facts are consistent with its being glycol, except' that its reducing value towards HIO_4 exceeds that calc. for $\frac{1}{2}$ mol. glycol. per mol. pentose. The nature of this substance will be further investigated.

Discussion of these results will be postponed until the fate of the 2-C moiety is cleared up. In the meantime, however, attention may be drawn to the requirement for inorganic P if fermentation is to proceed, suggesting the need for formation of a diphosphate, analogous to that found in hexose fermentation. Recently Dische [1938] has made the interesting observation that d-ribose combined in adenosine is phosphorylated by inorganic phosphate in blood haemolysate, and that the phosphorylated ribose is afterwards broken down to a number of products, including probably triosephosphate and glycollaldehyde or glycollaldehyde phosphate. Phosphorylation and fermentative cleavage of ribosephosphates may thus occur in animal cells also.

SUMMARY

d-Ribose-5-phosphoric acid is readily fermented by Lebedew macerate of dried beer yeast with the formation of 1 mol. alcohol, 1 mol. CO_2, 1 mol. free phosphoric acid and an as yet unindentified product.

Other pentose-5-phosphoric acids are very little fermented (d-arabinose and xylose esters were used). The rate of fermentation of ribosephosph. is similar to that of glucose or hexosemonophosphate (Robison ester), when allowance is made for the fact that these substances both give 2 mol. CO_2 and alcohol, instead of only the one given by ribosephosph. As would be expected, intact yeast cells hardly ferment ribose or its phosphoric ester.

An enzyme preparation free from coenzyme and phosphate required the addition of cozymase and phosphoric acid before fermentation set in. An activator (hexosediphosphate, acetaldehyde, glycollaldehyde) is needed to remove the delay in onset of fermentation, as with hexosephosphate fermentation in similar preparations.

The products of the reaction with the dried enzyme, cozymase, inorg. P, Mg and Mn salts, are the same as with Lebedew extract as far as the alcohol, CO_2 and inorg. P are concerned.

The experiments are being continued to determine the nature of the remaining product. It does not reduce alkaline Cu reagent, is not precipitated by neutral Pb acetate and appears to contain two or more adjacent unsubstituted hydroxyl groups.

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CCXIII. THE LACTIC DEHYDROGENASE OF LACTIC ACID BACTERIA

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THE occurrence of lactic dehydrogenase in lactic acid bacteria has not yet been demonstrated, although many authors have already proved the presence of the dehydrogenase in animal tissues and various micro-organisms.

The specificity of the form of lactic acid dehydrogenated by the enzyme has been pointed out by several authors. Meyerhof & Lohmann [1926] observed that *d*-lactic acid was more rapidly oxidized than the *l*-acid by yeast and animal tissues including muscle, liver, kidney and brain. The same conclusion was obtained by Banga *et al.* [1932] with muscle. Green & Brosteaux [1936] observed that the enzyme of heart muscle showed complete optical specificity in oxidizing only *d*-lactic acid, while *Bact. coli* oxidized the *l*-component more rapidly and the yeast preparation preferentially attacked the *d*-component.

In the present paper, the presence of lactic dehydrogenase was verified in all the kinds of lactic acid bacteria under investigation, and the enzymes of these bacteria were found to show complete optical specificities.

Methods

The various kinds of lactic acid bacteria were cultivated on 600 ml. koji extract containing 24–30 g. sugar and 30 g. $CaCO_3$ at 30° for 5 days. The bacterial cells were collected on the centrifuge after being separated from calcium carbonate by decantation. The cells were then suspended in 100 ml. water and strongly aerated. After being washed several times with water, the cells were finally suspended in 50 ml. of water and again aerated.

For the detection of dehydrogenase, the reduction time of methylene blue at 40° was measured anaerobically (less than 20 mm.) in a Thunberg tube into which 0·2 ml. of the bacterial suspension (in the case of *Lactob. sake* 0·4 ml. was used) was put with 0·2 ml. 5% Ca lactate solution and 1·8 ml. methylene blue solution prepared by mixing 8 ml. 0·05% methylene blue and 6 ml. of $0·2M$ phosphate buffer adjusted to *p*H 7·2.

Lactobacillus sake (d-*acid-former*)

When the experiments were carried out with *Lactob. sake* No. 84, it will be seen in Table I that reduction of methylene blue was observed in the presence

Table I. *Specificity of lactic dehydrogenase*

Donator	Reduction time of methylene blue (min.)		
	d-Acid-former	*l*-Acid-former	*dl*-Acid-former
d-Lactic acid	21	>180	25
l-Lactic acid	>180	11	13
dl-Lactic acid	20	17	15
Water (control)	>180	>180	>180

(1654)

of *d*- and *dl*-lactic acids. Therefore the bacterial cells were found to show complete optical specificity in oxidizing only *d*-lactic acid, as was already pointed out by Green & Brosteaux [1936] with heart muscle extract.

Leuconostoc mesenteroides (l-*acid-former*)

In contrast with the case of *Lactob. sake* mentioned above, only the *l*-component of lactic acid was available to *Leuconostoc mesenteroides* var. *sake* as donator (see Table I).

It is of interest that the optical specificities of the lactic acids dehydrogenated by these bacterial cells coincide with those of the acid produced by the bacteria themselves. Lactic dehydrogenase can therefore be classified into *d*- and *l*-enzymes.

Lactobacillus plantarum (dl-*acid-former*)

It will be seen in Table I that *Lactob. plantarum* sp. dehydrogenated all the optical components of lactic acid, although the *l*-component was attacked a little more rapidly.

Thus all the kinds of lactic acid bacteria mentioned above effected dehydrogenation of lactic acid in the presence of methylene blue. No oxidation of lactic acid was ever observed in the absence of methylene blue.

In order to dehydrogenate all the optical components of lactic acid, the presence of both *d*- and *l*-lactic dehydrogenases would not be absolutely necessary, since all forms of the acid could be attacked by any one of the dehydrogenases in presence of racemiase, with which the racemic form of lactic acid was always produced as has already been pointed out by us [1937].

It was found by us [1938] that racemiase in *Lactob. plantarum* was easily inactivated by treatment with acetone, while lactic dehydrogenase would not be inactivated by acetone, as was pointed out by Harden & Macfarlane [1931] with yeast. Therefore the experiments shown in Table II were carried out with acetone-dried bacteria in order to investigate the effect of racemiase upon the dehydrogenation of lactic acid.

Table II. *Effect of racemiase*

Lactob. plantarum dl-Acid-former	Reduction time of methylene blue (min.)		
	d-Lactic acid	*l*-Lactic acid	Water
Resting cells	38	18	>180
Acetone-dried cells	>180	20	>180

It will be seen in Table II that acetone-dried cells never attacked *d*-lactic acid, while *l*-acid was as easily dehydrogenated by it as by the resting cells.

The reason why *Lactob. plantarum* attacked all the forms of lactic acid was thus attributable not to the presence of both *d*- and *l*-lactic dehydrogenases, but to that of *l*-lactic dehydrogenase co-operating with racemiase.

The product of oxidation

The identification of pyruvic acid produced by oxidation of lactic acid with lactic dehydrogenase is not easy to accomplish, since decomposition of the pyruvic acid by carboxylase usually occurs or α-ketonoxidase is present in the dehydrogenase preparations.

In the present experiments, it was found that pyruvic acid could be directly isolated when lactic acid bacteria were used for the dehydrogenase.

(a) *Lactobacillus plantarum.* The experiments were carried out with seven large (100 ml.) Thunberg tubes. Each tube, containing 10 ml. 0·2 M Na *dl*-lactate, 10 ml. 0·2 M phosphate buffer (pH 7·2), 20 ml. 0·5 % methylene blue and 20 ml. bacterial suspension, was evacuated and kept at 40°. When the methylene blue was almost reduced, another 20 ml. of the methylene blue solution were added and the experiments were continued in exactly the same manner until decoloration of the dye was complete, which was attained in about 2 hr. throughout the whole of the experiments.

The decolorized solutions were collected, the dye adsorbed first with kaolin and then with active C. The clear solution thus obtained was acidified with H_2SO_4 and the pyruvic acid extracted by ether.

0·4 g. of yellowish acicular crystals, M.P. 184°, was obtained by the addition of phenylhydrazine hydrochloride solution to the aqueous solution of the acid after removal of the ether. After recrystallization the M.P. was found to be 192° which was identical with that of the phenylhydrazone of pyruvic acid. (Found: C, 60·49; H, 5·73 %. $C_9H_{10}O_2N_2$ requires C, 60·62; H, 5·62 %.)

The yield was 51 % of that calculated from the equation:

$$CH_3.CHOH.COOH + Mb = CH_3.CO.COOH + MbH_2$$
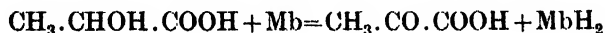
(b) *Lactobacillus sake.* The experiments were carried out in the same manner as was mentioned above, with two Thunberg tubes in which were placed 10 ml. 0·2 M Na *d*-lactate, 10 ml. 0·2 M phosphate buffer, 5 ml. 0·5 % methylene blue and 20 ml. of the bacterial suspension.

The solution was analysed after 5 hr., and 0·016 g. of the phenylhydrazone of pyruvic acid was obtained; yield found, 60 %.

(c) *Leuconostoc mesenteroides* var. *sake.* The same experiments as were instituted with *Lactob. sake* were carried out with *l*-lactate. The decoloration of the 15 ml. 0·5 % methylene blue in each Thunberg tube was attained within 6 hr. but the tubes were kept for a further 6 hr., in order to test whether pyruvic acid would be decomposed by the bacteria.

The yield of pyruvic acid was observed to be 58 %, since 0·049 g. of the phenylhydrazone was obtained from the two tubes.

No evidence of the production of acetaldehyde or acetic acid, which would be derived from pyruvic acid by carboxylase or α-ketonoxidase, was ever obtained in these experiments.

Thus it was clearly demonstrated that lactic dehydrogenase existed in all the lactic acid bacteria and that carboxylase could not be detected even in *Leuconostoc*, which converts glucose into lactic acid, alcohol and CO_2. This is in disagreement with the suggestion of Peterson *et al.* [1922] that the heterofermentation revealed by such types of bacteria as *Leuconostoc* would be caused by the presence of carboxylase.

SUMMARY

1. The occurrence of lactic dehydrogenase was verified with various kinds of lactic acid bacteria.

2. It was found that a *d*-acid-former (*Lactob. sake*) dehydrogenated only *d*-lactic acid and an *l*-acid-former (*Leuconostoc*) attacked only the *l*-acid. Lactic dehydrogenase is therefore considered to be a mixture of *d*- and *l*-enzymes.

3. The reason why a *dl*-acid-former (*Lactob. plantarum*) attacked all forms of lactic acid was not the presence of both kinds of lactic dehydrogenases, but the co-operation of racemiase with the *l*-enzyme.

4. The occurrence of carboxylase was never detected and pyruvic acid was easily isolated, in a yield of about 60%, as the oxidation product of lactic acid by any of the bacteria used.

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CCXIV.·A SURVEY OF ANTHOCYANINS. VI

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In earlier parts of this series [Robinson & Robinson, 1931; 1932; 1933; 1934; Lawrence *et al.* 1938] the nature of the anthocyanins present in the flowers, fruits, permanently pigmented leaves and autumn leaves of many species of plants has been described. To complete the analysis of the distribution of the principal anthocyanin types in the Angiosperms, a series of young leaves has now been examined. In young leaves coloration due to anthocyanins, disappearing with maturity, is a phenomenon which is probably more general than autumnal anthocyanin development, and the (approximately) 200 species, in 110 genera, tested were chosen to cover as wide a range of plants as possible.

As in autumn leaves, cyanidin saccharides predominate, being found in 93 % of the genera. In a few cases, other constituents interfered with the colour reactions and distribution, rendering it difficult to identify the glycosidal type definitely. But in those genera which contained cyanidin derivatives whose sugar types were determined, there were found:

31 % monoglycosides	30 % 3-biosides
50 % pentoseglycosides	9 % 3:5-dimonosides

A comparison of the figures with those derived from autumn leaves, flowers etc. will be made in a later communication.

Results

Figures refer to the numbers given by Hutchinson [1926] in his classification of the flowering plants, the first indicating the order and the second the family, e.g. 76–264 order Lamiales, family Labiatae. For completeness two plants have been included which were recorded previously, namely *Impatiens biflora* and *Crataegus oxyacantha fl. pl. rosea* [Robinson & Robinson, 1932].

Dicotyledons

76–264.	*Ajuga reptans* var. *atropurpurea*	o	67–238.	*Aster ericoides*	j
	Elsholtzia Stauntonii	b		*Silphium perfoliatum*	d
	Lamium purpureum	d		*Solidago aurea*	g
	Salvia nemorosa	o		*Sonchus oleraceus*	a
	S. officinalis	k	66–233.	*Abelia chinensis*	n
	S. virgata	m		*Lonicera Ferdinandii*	j
76–263.	*Callicarpa koreana*	g		*L. Maackii*	g
	Clerodendron foetidum	a		*L. Periclymenum*	j
	C. trichotomum	a		*L. Periclymenum* var. *belgica*	j
	Petraea volubilis	v		*L. pileata* var. *yunnanensis*	g
75–257.	*Bignonia Unguis-cati*	g		*Symphoricarpus orbiculatus* var.	g
75–256.	*Streptocarpus Rexii*	q		*conglomeratus*	
75–252.	*Pentstemon procerus*	l		*S. occidentalis*	g
	Veronica Chamaedrys	j		*Viburnum betulifolium*	o
74–250.	*Solandra Hartwegii*	c		*V. fragrans*	a
69–241.	*Ceratostigma plumbaginoides*	y		*V. Opulus* var. *sterile*	h
	C. Willmottianum	x		*V. rugosum*	d
	Limonium latifolium	A		*V. Tinus* var. *hirtum*	h
69–240.	*Dodecatheon Meadia*	o	65–230.	*Apocynum cannabinum*	d
	Steironema ciliatum	w	64–229.	*Jasminum officinale*	d

Monocotyledons

Acorus Calamus variegatus	d	Tradescentia bracteata		g
Alpinia Sanderae	d	T. virginiana		g
Lilium regale	d			

Hybrids

Cistus corbariensis	a	Phlox Coquelicot	o
Diervilla hybrida	d	Raphiolepis Delacourii	e
Lychnis Arkwrightii	c	Tritonia G. Davison	n
Philadelphus purpureo-maculatus	g		

Bouganvillaea, Mrs Butt, young leaves contained a nitrogenous anthocyanin with behaviour similar to that found in the bracts [Price & Robinson, 1937].

a   Cyanidin 3-monoside.
b   Cyanidin monoside.
c   Cyanidin 3-monoside + a little diglycoside.
d   Cyanidin 3-PG.
e   Cyanidin PG.
f   Cyanidin 3-PG + a little diglycoside.
g   Cyanidin 3-bioside.
h   Cyanidin 3-bioside + cyanidin 3-PG.
i   Cyanidin 3-bioside + cyanidin 3-monoside.
j   Cyanidin 3:5-dimonoside.
k   Acylated cyanidin 3:5-dimonoside.
l   Cyanidin diglycoside.
m   Acylated cyanidin diglycoside.
n   Cyanidin diglycoside + cyanidin monoside.
o   Cyanidin diglycoside + cyanidin PG.
p   Cyanidin saccharide with very high distribution.
q   Cyanidin 3-bioside + PG possibly containing some malvidin.
r   Peonidin 3-bioside + some cyanidin 3-bioside.
s   Peonidin 3:5-dimonoside + some peonidin monoside.
t   Peonidin 3:5-dimonoside.
u   Peonidin 3:5-dimonoside + some cyanidin 3:5-dimonoside.
v   Malvidin 3:5-dimonoside.
w   Malvidin PG.
x   Malvidin 3-PG + trace Fe^+ anthocyanin.
y   Malvidin 3-PG + some cyanidin 3-PG.
z   Malvidin 3-bioside + cyanidin 3-bioside.
A   Delphinidin 3-PG.
B   Delphinidin diglycoside + delphinidin PG.
C   Malvidin 3-PG + delphinidin 3-PG.

(PG = pentoseglycoside.)

ACKNOWLEDGEMENTS

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CCXV. A SURVEY OF ANTHOCYANINS. V

By WILLIAM JOHN COOPER LAWRENCE, JAMES ROBERT PRICE,
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EMPLOYING the methods previously described [Robinson & Robinson, 1931; 1932; 1933; 1934], further flower petals and permanently pigmented leaves have been examined in order to determine the anthocyanin type. Comments on these results together with those previously recorded will be submitted later. The only new points of technique are the following.

A characteristic property of peonin is that solutions in aqueous sodium carbonate are blue by transmitted daylight and reddish violet by transmitted artificial light. A number of cases have been encountered in which the presence of anthoxanthin and other substances have seriously modified the colour reactions and the distribution properties of anthocyanins, occasionally also of the derived anthocyanidins.

Repeated extraction with hot ethyl acetate or ethyl acetate after the addition of sodium chloride (free from magnesium chloride), or amyl alcohol in the case of diglycosides usually served to remove the interfering substances to a sufficient degree. Such processes must be followed by repeated extraction with benzene, or ether and then benzene, or exposure in a vacuum, in order to remove alcohol.

The autumnal colouring of leaves has been examined with the result that cyanidin saccharides are found to preponderate to a very large extent. The extracts were often contaminated with relatively large quantities of tannins and anthoxanthins and it was particularly difficult to determine the glucoside type. Moreover, it appeared that in a large number of the cases, a mixture of anthocyanins based on one anthocyanidin was present. In the presence of so much foreign material a rhamnoglucoside may simulate a diglucoside and a monoglucoside may behave like a rhamnoglucoside. Although the solutions were purified as far as possible, the results in the series of the autumn leaves are not so conclusive in regard to sugar type as those obtained with flower petals. Most of the anthocyanins produced in autumnal colouring are cyanidin 3-saccharides of various types. In this section the material is classified botanically, using Dr Hutchinson's system reversed so that the more highly developed plants are mentioned first in order; the first number indicates the order and the second the family [Hutchinson, 1926].

SUMMARY OF RESULTS

Flowers		Comments
Abutilon, hybrid, *Firefly*	e	
Agapanthus umbellatus	x	
Allium cyaneum	x	
Anagallis arvensis, scarlet	i	
A. arvensis, blue var.	y	
A. coerulea	z	
A. collina	i	
Anemone coronaria, blue violet var.	x	Partly methylated or mixed with cyanidin derivatives

Flowers		Comments
Anemone coronaria, red violet var.	h	With some delphinidin derivatives. The pigments of the anthers are monoglycosidic or pentoseglycosidic
Aphelandra nitens	i	With some pelargonidin diglycoside
Aubretia deltoidea, var. E. M. Crosfield	e	
Begonia socotrana	h	Acylated, colour reactions before alkaline hydrolysis were correct for 3 : 5-dimonoside
Beloperone guttata	c	In petals and bracts
Billbergia Sanderiana, blue petals	x	With trace of anthocyanin derived from cyanidin
B. Sanderiana, scarlet bracts	A	
Brachycombe iberidifolia	x	Much anthoxanthin
Browallia elata	B	Complex
Brunella grandiflora, violet red var.	e	Complex .
B. grandiflora, violet var.	x	Complex
Buddleia Davidii, var. *superba*	C	
B. Davidii, var. *magnifica*	D	
Camellia japonica, var. Lady Clare	a	Very high distribution, almost certainly a cyanidin complex 3-monoglycoside and would doubtless repay detailed investigation. Although the results were not entirely satisfactory, hydrolysis with alkalis gave an anthocyanin of lower distribution and hence the substance is probably an acylated cyanidin glycoside rather than a derivative in which the sugar is attached to the acyl group
C. sasnqua	a	Complex, probably identical with *C. japonica*
Campanula Portenschlagiana	B	
Cistus purpureus	E	Or complex anthocyanin resistant to hydrolysis
Correa speciosa, var. *ventricosa*	c	
Crocus asturicus	x	Kaempferol separated from the acid extracts
C. hadriaticus	x	
C. longiflorus	x	
C. nudiflorus	x	
C. pulchellus	z	
C. sativus	x	With a little complex diglycoside
C. speciosus	z	Extracts contained kaempferol
Dimorphotheca aurantiaca	k	Possibly pentoseglycoside carotenoids are present
Erica hyemalis	a	Much ivory anthoxanthin
Erodium macradenum	x	Methylated. The anthocyanidin is completely extracted by the delphinidin reagent, and not at all by the cyanidin reagent; but it is easily oxidized in strongly alkaline solution. Therefore it may be a new delphinidin derivative, unless a catalyst is present which facilitates the oxidation of malvidin. As this has never previously been observed, it seems more probable that we are dealing here with a new type of delphinidin derivative
E. Reichardii, var. *roseum*	x	
Escallonia edinensis	a	
Eschscholtzia californica, var. *The Mikado*	g	With anthoxanthin
Euphorbia fulgens	a	Much yellow anthoxanthin
Gentiana Lagodechiana	x	
Geranium Endressi	z	
Gilia capitata	x	
Godetia grandiflora, var. *Crimson Glow*	F	
Helianthemum nummularium, a var. with brick red double flowers	a	Much anthoxanthin
Hippeastrum aulicum	j	Co-pigmented
H. equestre	j	
Hydrangea macrophylla vars.	k	After careful purification the reactions and distribution ratios tallied with synthetic delphinidin 3-glucoside. The anthocyanins of red, blue, and violet flowers were found to be identical (correction of Part I)

Flowers		Comments
Hypoestes aristata	e	
Ipomoea Learii	z	
Iris unguicularis	B	
Ixora, hybrid, *Lord Williamson*	g	
Lapageria rosea	a	
Lilium ochraceum	c	
Linaria purpurea	x	
Luculia gratissima	z	Much anthoxanthin
Lychnis viscaria, var. *Blue Bouquet*	B	Indication of partial methylation
Lythrum salicaria, var. *superbum*, pink flowers	z	Strongly co-pigmented
L. salicaria, var. *superbum*, purple flowers	z	Strongly co-pigmented
L. salicaria, var. *Crimson Dwarf*	z	
Macleania punctata	i	
Malvaviscus conjattii	G	
Mesembryanthemum truncatum, var. *roseum*	K	
Monarda didyma, var. *Cambridge Scarlet*	H	Complex
Musa basjoo, bracts	c	
Naegelia cinnabarina	i	
Nemesia strumosa, scarlet var.	a	
Nierembergia coerulea	x	
N. frutescens	x	
Papaver Argemone, spots	g	With a little 3-monoside
P. Argemone, remainder of petals	G	With some pelargonidin 3-saccharide of higher distribution number
P. atlanticum	G	
P. commutatum, spots	g	
P. commutatum, remainder of petals	g	With little of pelargonidin or peonidin derivative
P. somniferum, spots	g	
P. somniferum, remainder of petal	g	As *P. commutatum*
Pelargonium inquinans	i	Noteworthy in a *P.* species
P. saniculaefolium	H	
Penstemon cordifolius	e	With anthoxanthin
Peristrophe speciosa	e	
Phacelia whitlavia	z	
Pisum sativum, purple var.	C	May contain delphinidin but no malvidin derivatives
P. sativum, violet var.	C	With a little malvidin derivative. In both vars. the outer parts of the flower are highly co-pigmented, the inner very little
Platycodon grandiflorum, var. *Mariesii*	x	Much anthoxanthin
Plumbago capensis	h	Much anthoxanthin obscures colour reactions, but the anthocyanin is probably a 3:5-dimonoside
Portulaca grandiflora	K	
Potentilla nepalensis		Acylated cyanidin saccharide. The colour reactions were those of 3-substituted cyanidin derivatives. On hydrolysis an acylated cyanidin was obtained
Pycnostachys Dawei	x	Acylated. Contains a trace of a cyanidin derivative
Raphiolepis Delacourii	a	Much anthoxanthin
Rhodochiton volubile, bracts	f	
Salvia Grahamii	J	Acylated, differing from salvin in that the solution in aqueous Na_2CO_3 is almost pure blue
S. neurepia	J	Acylated. Colour reactions tallied with those of salvin and monardin and differed from those of the anthocyanin of *S. Grahamii*
Silene Schafta	e	
Solanum crispum		Diglycoside of petunidin or delphinidin: the presence of cyanidin is not excluded if an oxidizing catalyst effective in alkaline solution is present
Sollya heterophylla	x	
Sphaeralcea australis	i	
Streptocarpus caulescens	z	Pure, no trace of anthocyanin giving a positive ferric reaction

Flowers		Comments
Streptocarpus Rexii	z	Containing a small amount of cyanidin di-monoside
Suaeda maritima	K	
Tibouchina semidecandra	z	Complex
Tropaeolum majus vars.		The presence of G was confirmed (previously recorded). Deeper coloured vars. contained mixtures in varying proportions of digly-cosidic anthocyanins based on cyanidin and delphinidin, ranging from almost pure cyanidin to almost pure delphinidin
Valeriana officinalis, a red var.	c	With a flavone derivative
Veltheimia viridifolia	e	Acylated
Verbena canadensis	e	
V. canadensis, var. *Drummondii*	e	
V. tenera	e	
V. erinoides	B	
V. venosa	B	With small amount of cyanidin diglycoside
V. radicans	B	
V. peruviana	i	
Veronica maritima	x	Complex
Vriesia Duvaliana, petals	x	
V. Duvaliana, bracts	z	Contaminated with a derivative of an antho-cyanidin exhibiting a ferric reaction under the usual conditions. The anthocyanin of the bracts could not be extracted even by boiling 1% HCl until the outer skin was removed, when the pigment was yielded to a cold solvent
Zauschneria californica, var. *latifolia*	i	
Zygocactus truncatus	K	

Leaves permanently coloured

Acalypha macrostachya	a	
Alocasia Lowii	g	Pigmented on underside only
Aphelandra nitens	x	May contain some cyanidin derivative and some monoglycoside. Pigment on underside of leaf only
Calathea insignis	L	
Codiaeum, hybrid, *Emperor Alexander III*	c	
Columnea Schiedeana	h	Only a trace of anthocyanidin was obtained by hydrolysis under the usual conditions, a sparingly soluble mauve precipitate being thrown down. Pigment on underside of leaf only
Cyanotis Kewensis	g	
Hoffmannia Ghiesbreghtii, var. *variegata*	f	
Hoya carnosa, var. *variegata*	c	Pigmented in chlorophyll-free parts only
Iresine Herbstii	K	
I. Lindenii	K	
Perilla nankinensis	e	Complex
Prunus persica, red-leafed var.	a	
Streptocarpus Wendlandii	g	Pigment on underside of leaf only
Strobilanthes Dyerianus	e	
Stromanthe Porteana, var. *variegata*	c	Strongly co-pigmented
Viburnum tomentosum, var. *plicatum*	a	With much anthoxanthin

Other organs

Cacao beans	a	
Cornus mas (Cornelian cherry)	i	In red berries
Euonymus yedoensis, capsules	a	
Pisum sativum, purple pods	x	
P. sativum, salmon pods	e	
Rubus idaeus, fruits	g	A number of varieties were examined and found to be mainly "g" together with smaller amounts of an anthocyanin derived from cyanidin of very high distribution which gave the same colour reactions as cyanidin 3-saccharides

ANTHOCYANINS FOUND IN LEAVES AFTER AUTUMNAL COLORATION

Species and varieties

76–264.	*Elsholtzia Stauntonii*	b		57–200.	*Acer circinatum*	a
76–263.	*Callicarpa Giraldiana*	e			*A. dasycarpum*	a
	C. japonica	e			*A. Davidii*	c
	C. koreana	e			*A. Ginnala*	b
69–241.	*Ceratostigma plumbaginoides*	b			*A. griseum*	c
	Limonium latifolium	b			*A. palmatum*	a
69–240.	*Steironema ciliatum*	k			*A. pseudoplatanus*	b
68–239.	*Swertia koreana*	b, o			*A. rubrum*	a
66–233.	*Diervilla Lonicera*	c			*A. rufinerve*	a
	Kolkwitzia amabilis	c			*A. Tschonoskii*	d
	Viburnum bitchiuense	h			*A. palmatum dissectum*	b
	V. dentatum	b			*A. palmatum septemlobum*	b
	V. dilatatum	a			*A. palmatum septemlobum elegans*	a
	V. fragrans	a			*A. palmatum roseo-marginatum*	b
	V. Lantana	d			*A. platanoides Reitenbachii*	d
	V. Lentago	d		57–205.	*Rhus canadensis*	b
	V. lobophyllum	a			*R. cotinoides*	a
	V. opulus	q			*R. cotinus*	a
	V. opulus sterile	a, n			*R. cotinus atropurpurea*	b
	V. orientale	a			*R. glabra laciniata*	b
	V. Sargentii	f			*R. Potaninii*	b
	V. setigerum	a			*R. toxicodendron*	k
	V. theiferum	a			*R. trichocarpa*	d
64–229.	*Fraxinus Ornus*	a, n			*R. typhina*	a
	Ligustrum obtusifolium	c			*R. typhina laciniata*	a
60–215.	*Enkianthus campanulatus*	a			*R. verniciflua*	a
	E. deflexus	f		55–194.	*Aegle sepiaria*	g
	E. perulatus	b		54–193.	*Vitis Coignetiae*	a
	Gaultheria procumbens	a			*V. Englemannii*	b
	Leucothoe Keiskei	b			*V. Henryana*	a
	Oxydendrum arboreum	f, t			*V. inconstans*	a
	Pieris Mariana	a			*V. pulchra*	e
	Rhododendron calendulaceum	b			*V. quinquefolia, var. Engel-mannii*	a, n
	R. canadense	a		51–173.	*Euonymus alata*	b
	R. mucronulatum	b			*E. americanus*	a
	R. occidentale	b			*E. Bungeanus*	a
	R. occidentale exquisita	a			*E. europaeus*	a
	R. pentaphyllum	b			*E. Maackii*	a
	R. ponticum	b			*E. nikoensis*	b
	R. reticulatum	a			*E. oxyphyllus*	b
	R. Schlippenbachii	b			*E. planipes*	a, n
	R. Vaseyi	a, n			*E. radicans*	b
	R. yunnanense	a			*E. sanguineus*	a
60–216.	*Gaylussacia baccata*	a			*E. verrucosus*	b, n
	G. dumosa	a			*E. Wilsonii*	b, n
	Vaccinium caespitosum	b			*E. yedoensis*	b
	V. corymbosum	a		48–162.	*Carpinus caroliniana*	b
	V. myrtillus	a			*Corylus americana*	b
	V. ovalifolium	a		48–163.	*Quercus alba*	b
	V. pennsylvanicum	b			*Q. coccinea*	f
	V. uliginosum	b			*Q. coccinea splendens*	h
	V. virgatum	b			*Q. glandulifera*	a
59–212.	*Aralia chinensis Mandschurica*	h, u		42–151.	*Corylopsis yunnanensis*	e
59–209.	*Cornus alba*	b			*Disanthus cercidifolius*	a
	C. alba Spaethii	h			*Fothergilla Gardenii*	h
	C. Amomum	f			*F. monticola*	g
	C. Baileyi	c			*Hamamelis japonica flavopur-purascens*	v
	C. florida	b			*H. japonica Zuccariniana*	w
	C. glabrata	b			*Liquidambar styraciflua*	a
	C. Koenigii	a			*Parrotia persica*	l
	C. Kousa	a		40–143.	*Amelanchier canadensis*	b
	C. Nuttallii	c			*A. florida*	b
	C. obliqua	c			*A. oblongifolia micropetala*	a
	C. Walteri	a				
59–211.	*Nyssa sylvatica*	a, n				

40–143.	*Aronia arbutifolia*	a	
	A. arbutifolia macrocarpa	d	
	A. melanocarpa	a	
	A. melanocarpa elata	b	
	Cotoneaster acutifolia	b	
	C. Franchetii	b	
	C. frigida	a	
	C. Henryana	b	
	C. horizontalis	a	
	C. rotundifolia	a	
	Crataegus acutiloba	b	
	C. Boyntoni	b, r	
	C. collina	b	
	C. Downingii	d, r	
	C. opposita	a	
	C. prunifolia	a	
	Fragaria indica	a	
	Malus coronaria elongata	b	
	M. ioensis plena	b	
	M. Tschonoskii	b	
	Neillia longiracemosa	d	
	Nuttallia cerasiformis	b	
	Photinia serrulata	a	
	Prunus Davidiana	a	
	P. glandulosa albo-plena	a	
	P. incisa serrata	b	
	P. pennsylvanica	h	
	P. Sargentii	a	
	P. Sieboldii	b, s	
	P. subhirtella	a	
	P. thibetica	b	
	P. yedoensis	d, o	
	Pyrus Calleryana	a	
	P. Pashia	a	
	Rosa blanda	a	
	R. nitida	a	
	R. rugosa	b	
	R. smoleschina	b	
	R. spinosissima	a	
	R. virginiana	b	
	Rubus allegheniensis	a	
	R. chroösepalus	b	
	R. cinnamomeus	a	

	Sorbus Folgneri	d	
	S. hupehensis	a	
	S. Sargentiana	a	
	S. serotina	d	
	Spiraea japonica ruberrima	a	
	S. nipponica	d, p	
	S. Sargentiana	a, n	
	Stephanandra incisa	a	
	Stranvaesia salicifolia	i	
	S. undulata	a	
39–139.	*Itea virginica*	a	
39–141.	*Ribes americanum*	j	
	R. cynosbati	b	
	R. odoratum	a	
39–142.	*Deutzia gracilis carminea*	d	
	D. scabra	h	
	Hydrangea quercifolia	c	
34–124.	*Eucryphia glutinosa*	a	
34–123.	*Hypericum patulum*	b	
	H. patulum Forrestii	b	
32–108.	*Stewartia pentagyna*	b, o	
	S. Pseudo-Camellia	b, o	
21–75.	*Punica Granatum*	a	
14–47.	*Bergenia cordifolia*	a	
5–19.	*Berberis acuminata*	b, o	
	B. aemulans	a	
	B. angulosa	a	
	B. Beaneana	a	
	B. consimilis	a	
	B. dictyophylla	a	
	B. Ferdinandi-Coburgii	f	
	B. Jamesiana	a	
	B. Julianae	a	
	B. replicata	b	
	B. Sargentiana	b	
	B. Thunbergii	b	
	B. Tischleri	b	
	B. Veitchii	d	
	B. virescens	a	
	B. yunnanensis	b	
	Mahonia japonica	b	
	Podophyllum Emodi	a	
1–7.	*Cercidiphyllum japonicum*	h	

Species hybrids

60–215.	*Rhododendron altaclarense*	b	
	R. viscosepala	b	
	R. occidentale × *calendulaceum*	b	
	R. malvatica × *Kaempferii*	b	
	"Jeannette"		
48–163.	*Quercus Leana* (= *Q. imbricaria* × *velutina*)	f	
40–143.	*Rosa calocarpa* (= *R. rugosa* + *indica*)	a	

Horticultural forms of unknown origin

60–215.	*Azalea*: "Raphael de Smet"	a	
	"Corneille"	a	
	"Il Tasso"	a	
	"Joseph Baumann"	a	
	KH 101 "Mrs A. Waterer"	a	
	KH 177 "Whitethroat"	a	
	KH 182	a	
57–200.	*Acer Davidii Forrestii*	b	

	A. japonicum aureum filicifolium	b	
	A. palmatum reticulatum	a	
	A. palmatum scolopendrifolium septemlobum Osakozuki	b	
	A. palmatum scolopendrifolium versicolor	b	
	A. platanoides laciniatum	b	
40–143.	*Pyrus communis*	c	

a Cyanidin 3-monoside.
b Cyanidin monoside.
c Cyanidin 3-pentoseglycoside.
d Cyanidin pentoseglycoside.
e Cyanidin 3 : 5-dimonoside.
f Mixed cyanidin saccharides, probably chiefly 3-saccharides.
g Cyanidin 3-bioside.

h   Cyanidin diglycoside.
i   Pelargonidin 3-monoside
j   Pelargonidin 3-pentoseglycoside.
k   Delphinidin 3-monoside.
l   Delphinidin 3 : 5-dimonoside.
n   These all contained amounts of diglycoside as well as the main anthocyanin.
o   The distribution of the anthocyanins in these cases was intermediate between monoside and pentoseglycoside.
p   The anthocyanin s largely pentoseglycoside, but contains a small amount of diglycoside. The aglycone from the pentoseglycoside fraction consists of cyanidin together with some anthocyanidin, in lesser amount, with a high distribution to the cyanidin reagent.
q   Largely cyanidin monoside containing some diglycoside which on acid hydrolysis gave a mixture of cyanidin and some anthocyanidin based on delphinidin.
r   The anthocyanins were monosides and the aglycones gave reactions indicating mixtures of cyanidin with peonidin. Cyanidin was certainly present, but the constituent with a high distribution to the cyanidin reagent may have been pelargonidin. Peonidin was considered more likely on account of marked blueing on dilution with absolute ethyl alcohol.
s   These two resemble *Spiraea nipponica* in that the aglycone consists of cyanidin containing some anthocyanidin with a high distribution to cyclohexanol-toluene. It may have been an acylated cyanidin, since unlike *Crataegus Boyntonii* and *C. Downingii*, after repeated washing of a cyclohexanol-toluene extract, the ferric reaction was still completely positive.
t   Mixture of cyanidin monoside with a cyanidin 3-saccharide of higher distribution.
u   The anthocyanidin was cyanidin, but the saccharide was of unusual type. The distribution (without salt) was nil, but on saturating with salt the pigment was partly extracted. This behaviour remained unaltered after purification (by repeated extraction with amyl alcohol and returning to 1% HCl and repeating the process). The colour reactions were dirty, but indicated a 3-saccharide.
v   Diglycosidic anthocyanins based mainly on malvidin together with a little cyanidin.
w   Similar to the above variety *flavo-purpurascens*, except that the amount of cyanidin present is greater.
x   Delphinidin diglycoside.
y   Malvidin 3-monoside.
z   Malvidin 3 : 5-dimonoside.
A   Pelargonidin 3 : 5-dimonoside and a cyanidin diglycoside in more or less equal proportions.
B   Delphinidin 3 : 5-dimonoside.
C   Petunidin diglycoside.
D   Petunidin 3-pentoseglycoside.
E   Petunidin 3-monoside.
F   Peonidin diglycoside.
G   Pelargonidin 3-bioside.
H   Pelargonidin 3 : 5-dimonoside.
J   Pelargonidin diglycoside.
K   Betanin type of pigment.
L   Delphinidin 3-pentoseglycoside.

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CCXVI. SPECTROGRAPHIC STUDIES ON THE ANTIMONY TRICHLORIDE REACTION FOR VITAMIN A

III. THE RELATION OF THE SPECTRAL ABSORPTION OF THE BLUE SOLUTIONS OF OILS TO THAT OF THEIR CONCENTRATES

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MEASUREMENTS of blue solutions of oils and their corresponding concentrates have been made by a number of authors using the tintometer [Coward *et al.* 1931; Dyer, 1933; Crews & Cox, 1934; Notevarp, 1935; Morgan & Pritchard, 1935], and also by some who have examined them both spectrographically and with the tintometer [Morgan *et al.* 1935].

Values for the ratio between the blue value of oils and that of their concentrates have been found between 1·16 (minimum found by Dyer [1933]) and 6·35 (maximum found by Morgan & Pritchard [1935]).

As calculated by means of the equations from Part I of these studies [Notevarp & Weedon, 1936], this corresponds to a minimum ratio between the 603 and 618 mμ bands of 0·65 and a maximum of 3·6. The ratios between the actually measured values for the two bands of Morgan *et al.* [1935] lie between 1·0 and 1·75.

We have shown in Part I that for purely physical reasons a concentrate gives a linear blue value[1] approaching twice that of an oil with the same absorption value. Higher or lower blue value ratios than a little under 2:1 must therefore signify that the absorptions at 603 mμ (oils) and at 618 mμ (concentrates) are not equal.

Higher blue value ratios are explained by inhibition of the reaction with the oil by the saponifiable fraction. In Part II of these studies [Notevarp & Weedon, 1938] this inhibition was shown to be considerable in all cases when the reaction was carried out in the usual way.

Loss of chromogen is the only reasonable explanation for 618 mμ values lower than the corresponding 603 mμ values. It follows therefore that as all hitherto published results for oils must presumably have been carried out under conditions favouring inhibition, all concentrate/oil blue value ratios as low as 1·0 or less show that substantial losses of chromogen must have occurred. Values above 2 or more indicate strong inhibition of the type found in unoxidized oils.

The object of this study was to investigate whether there exists a definite relation between the blue value and absorption of oils and concentrates.

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[1] To avoid misunderstanding "linear blue value" is defined as the value obtained by direct calculation to 18·18 g./l. from the concentration which gives a blue value of 6·0.

EXPERIMENTAL

The relation between oil and concentrate absorption determined with ordinary reagent

We measured $E_{603/618m\mu}$, blue value and $E_{328m\mu}$ for a number of oils both fresh and after some oxidation, and for their concentrates. The results are collected in Table I.

Table I

Oil no.	$E_{603m\mu}$	$E_{618m\mu}$	Ratio	Linear B.V.			$E_{328m\mu}$		
				Oil	Conc.	Ratio	Oil	Conc.	Ratio
15	0·21	0·26	1·24	2·5	4·9	1·96	0·19	0·10	0·53
3 unoxidized	0·54	0·94	1·74	5·9	16·0	2·70	0·48	0·40	0·84
3 max. by oxidation with air	0·90	0·94	1·05	9·6	16·0	1·67	0·48	0·40	0·84
2a	0·30	0·82	2·70	2·3	15·2	6·60	0·38	0·32	0·84
2b	0·70	0·82	1·17	7·8	15·2	1·95	0·38	0·32	0·84
4a	0·49	0·95	1·94	5·7	18·8	3·30	0·45	0·37	0·82
4b	0·85	0·95	1·12	9·8	18·8	1·92	0·45	0·37	0·82
1a	0·36	1·05	2·90	6·0	24·0	4·00	0·52	0·45	0·87
1c	0·88	1·05	1·19	10·4	24·0	2·30	0·52	0·45	0·87
7	1·80	2·35	1·30	16·0	30·0	1·88	0·90	0·77	0·86
18	17·8*	17·6	0·99	320·0	250·0	1·09	7·40	6·10	0·83

* Max. at 610 mμ.

The results confirm the deductions made in the introduction. After maximum absorption obtainable by oxidation with air was reached the $E_{618}/E_{603m\mu}$ ratio varied from 1·05 to 1·30. Unoxidized oils gave ratios from 1·74 to 2·9. One potent oil gave 0·99, probably because there was much less saponifiable matter in relation to the amount of chromogen, as the ultra-violet absorption showed that losses during preparation had not been excessive.

For reasons already explained, the ratio between the blue values was higher, ranging from 1·67 to 2·3, in the oils where maximum obtainable absorption had been reached. For unoxidized oils the range was 2·7 to 6·6. Oil No. 18 was an exception, as the properties of this oil approached those of a concentrate.

The figures are by no means unique, and are included merely to demonstrate that the relation between the blue values and 603–618 mμ absorption of oils and their concentrates varies considerably, not only in the case of unoxidized oils, but also for oils in which maximum absorption obtainable with air had presumably been reached. Apart from one oil of exceptionally low potency, the ratio between the ultra-violet absorption of the oils and the concentrates is constant within the limits of error of the method of determination and is in close agreement with the ratio demonstrated earlier by one of us [Notevarp, 1935]. The small loss of chromogen during preparation of the unsaponifiable fraction should therefore be approximately constant.

From these facts it must be concluded that there is no constant relation between the blue values, measured either optically or spectrographically, of oils and those of their concentrates when the ordinary reagent is used. For fresh oils strong inhibition makes the values much too low; the inhibition can be substantially reduced by oxidation with air, but even when maximum values have been reached by this method the fact that the concentrate values are definitely higher than the oil values shows that inhibition still exists, while the variations in the ratios demonstrate that no well-defined part of the inhibition is removed.

If, therefore, the 603/618mμ absorption is caused by vitamin A, we have further evidence of the view put forward in Part II of these studies, that the blue value as usually determined is not a true indication of the vitamin A potency of cod liver oils, even when the facts set out in Part I of these studies are taken into consideration.

If maximum absorption resulting from oxidation by air has been reached, however, and in Part II of these studies it was shown that this is probable in a majority of cases, the error is not much greater than the inherent inaccuracies of the blue value determination, a further explanation of the comparatively reliable blue values obtained in routine assays.

The relation between oil and concentrate absorptions using oxidizing reagent

Oils of low potency. $E_{603m\mu}$ and $E_{618m\mu}$ and the corresponding blue values were determined for cod liver oils using the oxidizing reagent containing 0·1 g./l. bromine described in Part II of these studies.

The oils were in various stages of oxidation and ranged from quite fresh ones to slightly rancid ones (oil 15). The results are recorded in Table II.

Table II

Oil no.	$E_{603m\mu}$	$E_{618m\mu}$	Ratio	Linear B.V. Oil	Conc.	Ratio	$E_{328m\mu}$ Oil	Conc.	Ratio
15	0·32	0·26	0·82	2·9	4·9	1·69	0·19	0·10	0·53
2	0·94	0·82	0·87	11·0	17·5	1·59	0·38	0·32	0·84
19	1·06	1·04	0·98	12·4	20·2	1·63	0·40	0·36	0·90
1b	1·32	1·15	0·85	15·0	24·2	1·61	0·52	0·45	0·86
20	1·60	1·52	0·95	21·0	35·0	1·66	0·61	0·56	0·92
21	1·80	1·65	0·92	24·2	39·0	1·62	0·72	0·64	0·89
Average ratio			0·90			1·63			0·88

The average ratio between $E_{328m\mu}$ for oils and $E_{328m\mu}$ for concentrates was about the same as the average ratio between $E_{603m\mu}$ and $E_{618m\mu}$, with about the same deviations from the average in the two cases. (Oil No. 15 was not included in the average for ultra-violet absorption ratio, as there was reason to believe that the value obtained for this oil was too high owing to the presence of substances other than vitamin A). The blue value ratio, as would be expected, was higher (average 1·63), with about the same deviations as the other ratios. This shows that when the bromine reagent is used there is a definite relation between the SbCl$_3$ reaction values for oils and their concentrates, corresponding to the well known relation between the ultra-violet absorption values. The effect of preparation of the concentrates is a loss in $E_{603/618m\mu}$ of about 10%, together with a shifting of the band; these two effects together determine the blue value ratio, according to the relations discussed in Part I of these studies.

Oils of potency above the average. Three cod liver oils of potency considerably above the average were measured by means of the bromine reagent. Unfortunately no fresh samples were available, as cod liver oils of such potency are scarce and are only available when oils from single livers are prepared. The results are recorded in Table III.

The first two oils showed lower $E_{603/606m\mu}$ than E_{618} values, indicating that the inhibition by the rancid oil was not completely removed. The most potent oil showed the usual relation, while the blue value ratio was considerably lower than

for less potent oils. This is because the absorption band was no longer at 603 mμ, but had moved to 606 mμ, indicating that the properties of the oil were approaching those of a concentrate.

Table III

Oil no.	$E_{606\,m\mu}^{608\,m\mu}$	$E_{618\,m\mu}$	Ratio	Linear B.V.			$E_{328\,m\mu}$		
				Oil	Conc.	Ratio	Oil	Conc.	Ratio
17	4·5	5·3	1·17	51	84	1·64	2·0	1·7	0·85
16	13·4	15·2	1·13	190	296	1·55	5·5	4·7	0·85
18	19·0	17·6	0·93	300	350	1·17	7·4	6·1	0·83

Shifting of the band from 603 to 618 mμ

No experimentally proved explanation of the shifting of the main absorption band of the SbCl₃ reaction from 603 to 618 mμ has come to our notice. The occurrence of vitamin A in an esterified form in oils, and as a free alcohol in concentrates, may be an explanation, while we ourselves have ascribed the
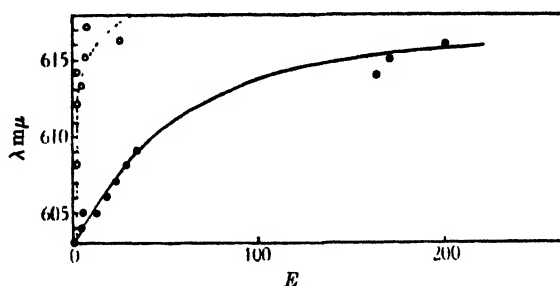
Fig. 1. Alteration in wave-length with increasing $E^{10g/l}$.
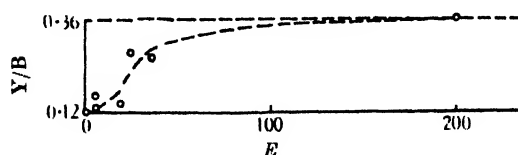
Fig. 2. Alteration in Y/B ratio with increasing $E^{10g/l}$.
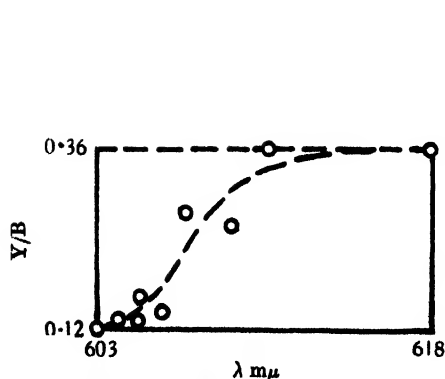
Fig. 3. Alteration of Y/B ratio with increasing wave-length.
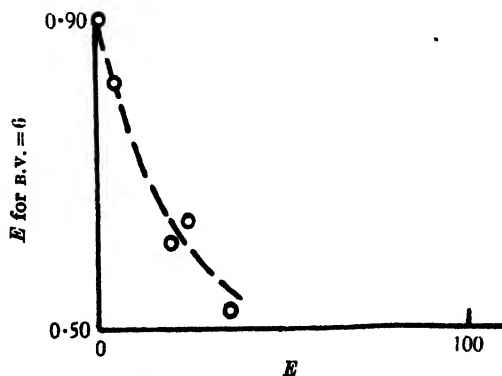
Fig. 4. Alteration of E for B.V. = 6 with increasing $E^{10g/l}$.

shifting to the change in the solvent effected by the unsaturated glycerides of the oils [Notevarp & Weedon, 1936]. If the latter explanation is correct, the maximum should move gradually towards $618\,m\mu$ with rising E values.

Table IV

Oil no.	No. times fortified	E_{max}	$m\mu$	Ratio Y/B	$E_{B.V.-1}$	$E_{B.V.-6}$
15	—	0·32	603	0·12	6·5	0·90
15	21	5·30	605	0·16	6·8	0·84
15	101	24·00	607	0·27	8·6	0·64
17	—	4·50	604	0·13	—	—
17	41	200·00	616	0·36	—	—
16	—	13·40	605	0·12	7·2	0·80
16	11	163·00	614	—	—	—
18	—	19·00	606	0·14	8·9	0·61
18	2	35·00	609	0·26	10·0	0·52
14 (halibut)	—	170·00	615	—	—	—

As an experimentally proved explanation would seem to be of interest, both theoretically and as a basis for a better understanding of the relation between the blue value and chromogen content of oils, we have measured absorption, location of bands and blue values of oils and of the same oils fortified with their own concentrates, using the bromine reagent. We have also measured the ratio of yellow to blue matching in the Tintometer, as this indicates the location of the band, while on the other hand the relation between this ratio and the location of the band is unknown.

The results have been collected in Table IV together with the E values corresponding to a blue reading of 6, shown graphically in Figs. 1 and 2, 3 and 4. In Fig. 1 are also shown some values obtained by using the ordinary reagent.

DISCUSSION

Comparison of the three methods for the determination of vitamin A in oils and their concentrates, i.e. blue value, absorption of $SbCl_3$ blue solution and ultra-violet absorption, shows that with the ordinary reagent there is in all cases definite inhibition of the $SbCl_3$ reaction with oils, making absorption values and consequently blue values too low. The degree of inhibition varies for different oils, even when the major portion of the inhibiting factors has been removed by oxidation with air. This suggests strongly that although the blue value of oils obtained by the use of ordinary reagent in a majority of cases gives a good indication of the vitamin potency, the varying degree of inhibition is a source of error which cannot be controlled when such reagent is used, and may, in the case of fresh oils, lead to very considerable errors.

With the use of the bromine reagent the relation between the absorption of $SbCl_3$ blue solutions of oils and their concentrates is constant within the limits of error of the determinations for normal oils of low to medium potency and is the same as the corresponding relation between the ultra-violet absorptions. For such oils, therefore, the inhibition of the $603\,m\mu$ band is practically completely removed, consequently the ratio between the blue values is constant and about 1·6, in accordance with the conclusions reached in Part I of these studies.

The values for three oils of higher potency do not allow a very definite interpretation, probably because the oils were of uneven freshness. A considerable portion of the inhibition must have been removed, but although $E_{603m\mu}/E_{618m\mu}$

is still above 1 for two of the oils, the B.V. ratio is about the same as for ordinary oils with an E ratio of about 0·9. Complete removal of the inhibition would therefore have resulted in a lower B.V. ratio indicating shifting of the bands towards 618 mμ. This is in fact the case with the third oil, where the E ratio is normal.

Table IV and Figs. 1–4 throw light on the mechanism of the shifting of the band from 603–618 mμ. A greater number of determinations would have been desirable, especially as the accuracy of determination of both wave-length of the maximum absorption and $Y:B$ ratio is not very great. Nevertheless, the values shown demonstrate that there is a continuous change in both wave-length and $Y:B$ ratio with rising E values.

The shifting of the band is therefore due to some gradually changing influence, most probably the concentration of the glycerides, as we have suggested earlier. There should therefore exist a simple mathematical relation between the distance the band has moved, $\Delta\lambda$ (in mμ), and the concentration of glycerides, which again is inversely proportional to the chromogen concentration, i.e. to E. Such a relation would be of a tangential type. In Fig. 1 the curve corresponds to a function

$$E = 50 \, tg \, 6\Delta\lambda$$

where $6\Delta\lambda$ is expressed as degrees. As $\Delta\lambda$ can vary from 0 to 15, the function varies from 0 to ∞. It will be seen that the measured values are grouped sufficiently close around the curve to make the simple tangential relation highly probable.

Although the three other figures contain only a small number of values, they nevertheless show that for E values somewhere about 50, or, according to Fig. 1, for maximum wave-lengths above 610 mμ, the Y/B ratio and the $E_{B.V.=6}E$ ratio approach those of a concentrate. This means that if insignificant inhibition of the main band is assumed, linear blue values above 5–600 should be proportionate to the content of chromogen, as is the case with concentrates.

The curve obtained with the ordinary reagent in Fig. 1 indicates that the shifting of the band is more rapid than when the bromine reagent is used. On the other hand, the ordinary reagent involves the inhibition which is removed by the bromine; the picture is thus complicated and it would seem of little interest to study this relation closer.

SUMMARY

By measurements of the SbCl₃ absorption of oils and their concentrates with the ordinary non-oxidizing reagent, no definite relation was found between oil and concentrate absorption and blue values, even when maximum absorption of the oils by oxidation with air had been reached. The oil absorption values were lower than those of the corresponding concentrates, indicating that there remained inhibiting factors in varying amounts.

When the bromine reagent was used the relation between oil absorption and concentrate absorption values was constant for normal oils, and corresponded closely to the relation between the ultra-violet absorption values. The blue value ratio was also constant, but in accordance with the facts set out in Part I of these studies the blue values of the concentrates were higher than those of the oils.

Bromine reagent would therefore appear to eliminate the difference between the SbCl₃-reaction with oils and their concentrates except the shifting of the absorption maximum. If the laws governing the latter are known, there should be no reason to isolate the unsaponifiable matter to obtain a reliable blue value for normal oils.

Measured values for the relation between wave-length and E indicated that there is a continuous movement of the band from 603 mμ towards 618 mμ with increasing E. If the shifting is caused by the concentration of glycerides the shifting should follow a tangential function; this is shown to be probable. The ratios between yellow and blue matching and the E value for a blue reading equal to 6 probably also change continuously. The curves found for all relations show that the properties of a concentrate are approached from about $E = 50$; above this value, therefore, the linear blue values should be proportionate to the chromogen content.

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CCXVII. THE PROTEOLYTIC ENZYMES OF SPROUTED WHEAT. III

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IN two previous communications the author [1936, 1, 2] has described the chief properties of the proteinase and dipeptidase found in aqueous extracts of sprouted wheat. The present paper is a continuation of these studies and deals primarily with the action of the proteinase on gelatin, ovalbumin and on the principal proteins of wheat, namely, gliadin and glutenin, both separately and in the form of gluten.

EXPERIMENTAL

The Sørensen method of formaldehyde titration in two stages, (*a*) and (*b*), again formed the basis of investigation, enzyme activity being recorded in terms of $N/20$ NaOH increase.

Substrates. The gluten, gliadin and glutenin used as substrates were prepared from a sample of untreated Canadian flour. The gluten was obtained from a dough made by mixing 100 g. flour with 55 ml. tap water. The dough was first immersed in tap water at 18° for 1 hr. and was then kneaded under a stream of tap water until all starch, etc. had been removed. After a final washing in distilled water and the removal of surplus water the gluten mass was cut into small pieces and mixed with sufficient $N/5$ acetic acid to yield a final gluten concentration of 10%, allowance being made for the water retained by the gluten. This water content was determined by drying a sample for 24 hr. at 100°. Gluten, of course, consists almost entirely of gliadin, glutenin and water, the two proteins together forming about 34% and the water about 66% of the mass, although small amounts of lipins, mineral matter etc., remain firmly adherent.

The gliadin and glutenin were prepared simultaneously by a modification of methods proposed by Troensegaard [1931] for gliadin and by Damodaran [1931] for glutenin. Wet gluten, obtained as described above, was cut into small pieces and the gliadin extracted by several changes of 70% ethyl alcohol at 28°. The dissolved protein was precipitated by concentration of its solution under reduced pressure at 30° and purified, firstly by treatment with ether to remove fats, lipins etc., secondly with distilled water to remove mineral matter etc., and finally by re-solution in 70% alcohol and precipitation by concentration again. Dehydration with alcohol in gradually increasing concentration up to absolute alcohol completed the process.

Treatment of the air-dried residue from the gliadin extraction with 0·2% NaOH yielded a solution of glutenin which was precipitated by adjusting the pH to 6·8–7·0 (the isoelectric point of glutenin) by addition of acetic acid. The precipitate was purified by repeated dissolution in 0·2% NaOH and subsequent precipitation with 0·4% acetic acid at pH 6·8–7·0. The protein precipitate was washed with distilled water by decantation until free from acid, then with absolute alcohol and finally with ether. Both preparations were thereafter kept *in vacuo* over P_2O_5.

RESULTS

Determination of pH optimum for gelatin. A freshly prepared 10 % aqueous solution of Gold Leaf gelatin was used as substrate. The corrected curve shown in Fig. 1 has an optimum at pH 5·1. In the same figure are included the experimental test and control curves from which the corrected curve was calculated by difference. The control curve was determined on mixtures containing buffers and enzyme extract only.
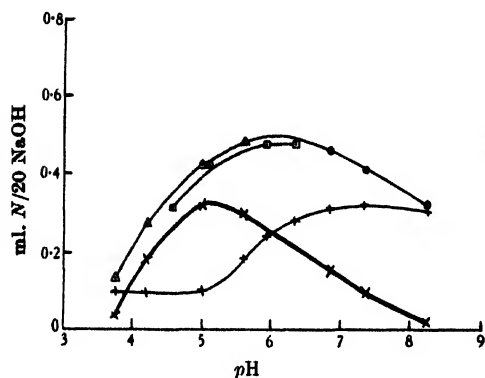
Fig. 1.   Fig. 2.
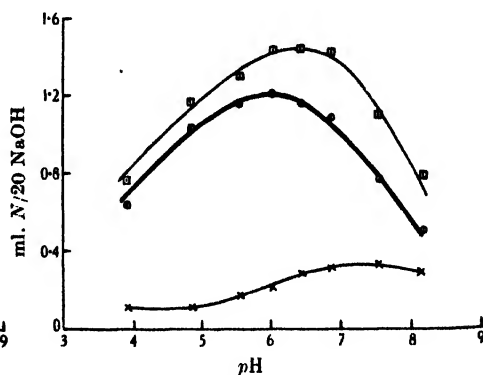
Fig. 1. *Effect of variation in pH on gelatin hydrolysis.* Conc. of gelatin in reaction mixtures: 2·0 %. Enzyme dilution 2 : 7. $M/7$ acetate, $M/7$ KH_2PO_4-NaOH and $M/7$ K_2HPO_4-NaOH buffers. Titres as ml. $N/20$ NaOH per 5 ml. sample. Reaction 4 hr. at 40°. ▲ Acetate buffers. ☐ KH_2PO_4-NaOH buffers. ⊙ K_2HPO_4-NaOH. + Control curve. × Corrected curve.

Fig. 2. *Effect of variation in pH on gluten hydrolysis.* Conc. of gluten in reaction mixture: 3·0 %. Enzyme dilution 2 : 7. $M/7$ potassium phosphate-NaOH buffers. ☐ Test curve. ⊙ Corrected curve. × Control curve.

Action of proteinase on ovalbumin. Over the range covered by acetic acid—sodium acetate buffers (pH 3·75–5·7) no increase in titre above that of the control value was observed after 4 hr. at 40°. This result was confirmed after 4 hr. and 24 hr. respectively, at 40° by Harris's [1923] method of titration in 85 % alcohol using thymolphthalein as indicator. As substrate, a filtered 5 % solution of dried egg albumin (Merck) in $N/5$ acetic acid was used.

Determination of pH optimum for gluten. The effect of variation in pH on gluten hydrolysis by wheat proteinase was determined after 4 hr. at 40°. The results in Fig. 2 show that the corrected curve has an optimum range of pH 5·6–6·4, with a mean value of pH 6. All reaction mixtures contained a considerable amount of precipitated gluten proteins.

Effect of NaCl on gluten hydrolysis. In view of the inhibitory effect of certain concentrations of NaCl on edestin hydrolysis by wheat proteinase [Mounfield, 1936, 1, Fig. 7], parallel experiments were conducted at pH 6 with gluten (final conc. 2·4 %) as substrate. With increasing concentrations of NaCl up to 0·25 M the difference from the control value without salt did not exceed 0·04 ml. $N/20$ NaOH which is below the limit of experimental error. As in the preceding experiment all reaction mixtures contained a curdy white precipitate of gluten proteins, the amount of which was not, however, increased by increasing salt concentration. Sodium chloride up to 0·25 M is therefore without influence on gluten hydrolysis by wheat proteinase.

Relation between initial reaction velocity and gluten concentration. Variation in gluten concentration produced the results indicated by the upper and lower lines in Fig. 3. The increases for total titre $(a+b)$ and titre (b) did not bear a constant ratio to each other as expected and both are therefore reproduced in the figure after deduction of control values without gluten. With increased concentration of gluten there was a corresponding increase in the amount of precipitate in the reaction mixtures.
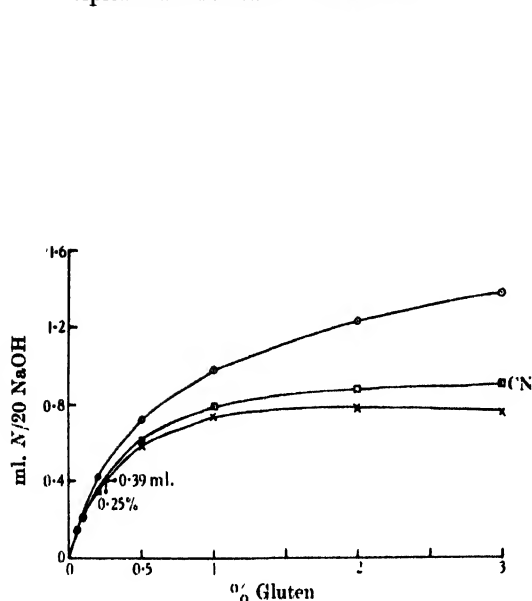
Fig. 3.

Fig. 4.

Fig. 3. *Effect of gluten concentration.* Enzyme dilution 2 : 7. $M_,7$ potassium phosphate-NaOH buffer at pH 6. Reaction 4 hr. at 40°. ⊙ Total titre without cyanide. ⤬ Titre (b) without cyanide. ▣ Titre (b) with cyanide.
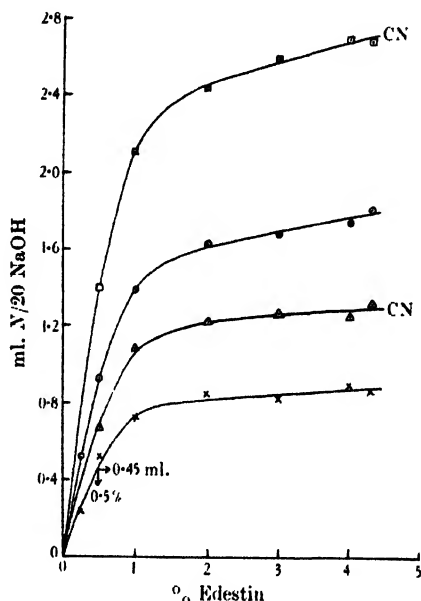
Fig. 4. *Effect of edestin concentration.* Enzyme dilution 2 : 7. $M/7$ acetate buffers at pH 4·1. ⊙ Total titre without cyanide. · Titre (b) without cyanide. ▣ Total titre with cyanide. ⌀ Titre (b) with cyanide.

The curve for titre (b) reaches a maximum at a gluten concentration of approximately 1·5 % whereas the curve for total titre continues to rise over the whole range of substrate concentration examined.

Relation between initial reaction velocity and edestin concentration. The effect on proteinase activity of variation in edestin concentration differs to some extent from that obtained with gluten as substrate. The ratio between the increases in total titre and titre (b) (see Fig. 4), is almost constant throughout at 2 : 1.

Effect of NaCN on hydrolysis of edestin at varying concentrations of the latter. Increases in titre in the presence of cyanide may conceivably be due to a more complete hydrolysis of the cleavage products rather than to a simple acceleration of reaction velocity. Data which appear to throw some light upon this problem were obtained by redetermining the curves showing the effect of edestin concentration on proteinase activity in the presence of a constant concentration of cyanide, i.e. 0·01M (see Fig. 4). The curves for total titre and for titre (b) in the presence of cyanide are proportionately higher in both cases than are the corresponding curves in the absence of cyanide.

Influence of cyanide upon proteinase activity using gluten as substrate. The addition of cyanide to mixtures containing gluten as substrate caused no activation of the wheat proteinase as measured by increase in total titre. There was, however, some alteration in the ratio of titre (*a*) to titre (*b*): the latter was increased slightly at the expense of the former, total titre remaining the same as in the absence of cyanide. The middle line in Fig. 3 represents the effect upon titre (*b*) of a constant concentration (0·01 *M*) of cyanide when the concentration of gluten is varied.

Effect of cyanide on control values. During the determination of control values (without added substrate) in connexion with the foregoing cyanide experiments it became evident that at certain *p*H values the cyanide caused not only a small but definite increase in total titre over and above the corresponding control value without cyanide but also an appreciable alteration in the ratio titre (*a*) to titre (*b*). The normal control mixtures consisted of enzyme extract + buffer, with water in place of substrate solution: the cyanide controls contained NaCN to a final concentration of 0·01 *M*. In Table I are presented a few typical values for titres (*a*) and (*b*) at different *p*H values with and without cyanide.

Table I

*p*H value		4·11	5·23	6·02	7·15	8·08
Without cyanide	Titre (*a*)	0·02	0·02	0·01	0·02	0·02
	Titre (*b*)	0·08	0·10	0·20	0·29	0·27
	Total	0·10	0·12	0·21	0·31	0·29
With cyanide	Titre (*a*)	0·00	0·00	− 0·04	− 0·08	− 0·09
	Titre (*b*)	0·11	0·14	0·28	0·44	0·46
	Total	0·11	0·14	0·24	0·36	0·35

The influence of cyanide is first to produce a slight increase in total titre and secondly to enlarge titre (*b*) at the expense of titre (*a*). Both these changes are more pronounced at the more alkaline reactions.

Action of wheat proteinase on gliadin and glutenin. Gliadin and glutenin prepared in the manner already outlined do not appear to be affected to any great extent by wheat proteinase. Over the range *p*H 3·75–8·2 no increases in titre greater than those of the control could be detected except at about *p*H 5. At this point a slight optimum was discernible in the case of gliadin. Substrate concentrations of 2·0 % in *N*/5 acetic acid were employed. Doubling the concentration of protein produced no further effect upon the titre increase. These results were confirmed on four occasions with separate preparations of gliadin and glutenin.

In view of any possible modification of the glutenin by the NaOH used in its preparation the action of wheat proteinase was determined on a sample of glutenin prepared as already described with the exception that solution in 0·2 % NaOH and precipitation with 0·4 % acetic acid were omitted, the air-dried residue being simply dehydrated with increasing concentrations of alcohol. The proteinase was found capable of attacking this substrate and although the extent of scission was by no means as great as in the case of gluten it was sufficiently great to reveal a slight optimum at approximately *p*H 5·5.

DISCUSSION

The investigation of the effect of wheat proteinase on proteins other than edestin reveals a somewhat unexpected range of specificity. Gelatin and gluten are readily attacked whereas ovalbumin and the constituent proteins of gluten—

gliadin and glutenin—are not. Gelatin shows an optimum at pH 5·1 (see Fig. 1), a value which is close to the isoelectric point of the protein, pH 4·9 [Pauli & Modern, 1925]. Papain [Willstätter *et al.* 1926], yeast proteinase [Willstätter & Grassmann, 1926] and malt proteinase [Lüers & Malsch, 1929] also attack gelatin optimally at pH 5. These facts suggest that plant proteases, generally, hydrolyse the unionized form of gelatin.

It will be observed from Fig. 1 that phosphate used as buffer has a slight retarding influence on the rate of proteolysis. The phosphate curve is, however, parallel over its whole range with the acetate one. The importance of the control curve correction is made particularly manifest in Fig. 1, an apparent optimum of pH 6 being reduced thereby to one of pH 5·1. It should be borne in mind, however, that the use of the correction curve (obtained from mixtures of buffer and fresh enzyme extract only) is based on the assumption that on the addition to the control mixture of another substrate such as gelatin the latter will not be preferentially attacked by the proteinase, an assumption which may not be entirely justified.

The behaviour of wheat proteinase towards ovalbumin finds again a parallel in the behaviour of papain [Willstätter & Grassmann, 1924], yeast proteinase [Grassmann & Dyckerhoff, 1928] and malted barley proteinase [Linderstrøm-Lang & Mill, 1929] towards this substrate; in no instance is the native protein hydrolysed. The albumin is however attacked by yeast proteinase which has been activated by cyanide while the denatured protein is degraded by papain without cyanide activation [Willstätter *et al.* 1926]. The above evidence of absence of hydrolysis of ovalbumin was secured either by alcohol or formaldehyde titration of the end products of hydrolysis. On the other hand, by measuring the disappearance of substrate, as indicated by the loss of coagulability of the ovalbumin, Hopkins & Kelly [1931] were able to demonstrate an optimum of pH 3·3–3·6 for the action of malted barley proteinase on the native protein. It is conceivable that the chemical changes accompanying the loss of coagulability are not of sufficient magnitude to influence a formaldehyde or alcohol titre. Northrop [1922], for example, obtained different results on measuring the effect of substrate concentration on the tryptic digestion of casein according to whether he measured the rate of disappearance of casein or that of amino-nitrogen liberation.

From the curves in Fig. 2 it is clear that wheat gluten is acted upon by the proteinase present in aqueous extracts of sprouted wheat, the reaction having an optimal range of pH 5·6–6·4 with a mean value of 6·0. This figure is slightly to the acid side of the isoelectric points of gliadin and glutenin as reported by Tague [1925], who found a value for gliadin of pH 6·5 and for glutenin of pH 6·8–7·0. The choice of gluten as a substrate is of course open to criticism: gluten is neither a pure protein nor simply a group of proteins. It contains in addition to gliadin and glutenin small quantities of fats and lipins, carbohydrate, mineral matter etc. A typical analysis of dried gluten is given below [Norton, 1906]:

Fat or ether extract	4·20
Carbohydrate	9·44
Fibre	2·02
Mineral matter	2·48
Gliadin	39·09
Glutenin	35·07
Globulin (10% NaCl extract)	6·75
	99·05

Gluten was nevertheless employed as such, firstly on account of its unique position among proteins, secondly, because it is presumably the natural substrate for wheat proteinase, and thirdly, on account of its considerable technical importance. That the results shown in Fig. 2 are due to hydrolysis of the principal gluten proteins and not to the decomposition of concomitant substances such as fats and lipins is proved by the method of their determination: increases in titre (b) by formaldehyde titration can be due only to the formation of amino-acids or closely related compounds. Further, although there arises the possibility of the hydrolysis of other proteins such as wheat globulin or leucosin which remain in the mass of gluten, nevertheless the extent of this hydrolysis could have but little influence upon the final result on account of the extreme dilution of these proteins in the final mixtures. For example, assuming the amount of globulin in the sample of dry gluten used in the present experiments to have been 7 %, the concentration of the globulin in the final reaction mixtures could not at any time have exceeded 0·21 %. This corresponds to a final crude gluten percentage of 3. If then the curves in Fig. 3 are assumed to be the outcome of, say, wheat globulin hydrolysis, then not only are titre increases of considerable magnitude obtained at substrate concentrations of 0·21 % but appreciable increases still accrue at concentrations as low as or even lower than 0·007 % (corresponding to a gluten percentage of 0·1). Judging from the data obtained with edestin and gelatin such an interpretation is highly improbable.

Gluten hydrolysis by wheat proteinase does not appear to be affected by sodium chloride in concentrations up to 0·25 M, nor is the amount of substrate precipitate present in each mixture increased by the salt addition. Edestin, on the other hand, is precipitated by sodium chloride in acid solution and the rate of hydrolysis of edestin in the presence of this salt has been shown to depend upon the amount of protein remaining unprecipitated [Mounfield, 1936, 1]. The apparently obvious explanation that by precipitation the edestin is removed from the sphere of activity of the enzyme cannot, however, be accepted as the sole reason for the inhibitory effect, since precipitation of edestin can occur in certain mixtures containing acetate buffers only, particularly at about pH 5, without an equivalent reduction in rate of hydrolysis [Mounfield, 1936, 1, Fig. 1]. The edestin precipitated in sodium chloride solutions does not therefore appear to be identical with that formed by simple adjustment of an edestin solution to its isoelectric point. This suggests the formation of an edestin-sodium chloride complex which is not attacked by the proteinase. That the latter enzyme is capable of directly hydrolysing precipitated substrate is proved still further by the experiments on the relation between initial reaction velocity and gluten concentration. At all concentrations from 0·05 to 3·0 % the gluten appeared to be completely precipitated, yet with increase in gluten concentration there was continued increase in total titre (see Fig. 3).

Figs. 3 and 4 depict the results of parallel experiments on the variation in initial reaction velocity with substrate concentration using gluten and edestin, respectively. In the case of gluten the titre (b) curve reaches a limiting value at a substrate concentration of approximately 1·5 %, while for edestin the curve becomes almost horizontal at approximately 2 % substrate. The latter curve was published in an earlier communication [Mounfield, 1936, 1] but is here reproduced for convenience in reference. The substrate concentration at which half the limiting velocity is reached should be numerically equal to the Michaelis constant, K_m, a characteristic of the enzyme. For edestin this has already been reported as occurring at about 0·05 %. From Fig. 3 the value for gluten is seen to be approximately 0·25 % although in view of the complex nature of this

substrate and the probable interference of dipeptidase activity (see below) the precise meaning to be attached to this information is rendered even more obscure than in the case of edestin.

The curve in Fig. 3 showing increase in total titre $(a+b)$ with variation in substrate concentration does not run parallel with that for titre (b), the ratio titre (a) : titre (b) increasing with increasing substrate concentration. In Fig. 4 however the curves for total titre and titre (b) approximate more closely in shape, the ratio total titre : titre (b) being almost constant at 2 : 1. In neither case is a limiting value reached within the limits of the experiment.

In this connexion it should be borne in mind that titre (b) represents principally the increase during hydrolysis in amino-acid-N together with a portion of the polypeptide amino-N while total titre includes also that portion of the polypeptide amino-N not measured in titre (b) and also any carboxyl groups liberated, for example, by ester scission [Richardson, 1934; 1935; Grünhut, 1919]. Ester scission is unlikely in the present instance and in any case would be very largely eliminated by the deduction of control values so that increases in titre (a) or titre (b) may safely be assumed to result from hydrolysis of protein only.

Now the mean pK' of most peptides is about 8·3 while that of amino-acids is about 9·6 [Richardson, 1934] so that peptide formation alone (if such should occur) would lead to increases in both titre (a) and titre (b). Amino-acid production on the other hand would result in increase in titre (b) without appreciable rise in titre (a). The distinction is of course by no means quantitative but merely indicates a tendency. The hydrolysis therefore of, say, glycylglycine $(pK' = 7·75)$ to glycine $(pK' = 9·75)$ or of leucylglycine $(pK' = 7·83)$ to glycine and leucine $(pK' = 9·6)$ should cause a decrease in titre (a), a comparatively large increase in titre (b) and a small increase in total titre. These changes were observed experimentally and are discussed below.

Thus the continued rise in total titre with increasing substrate concentration in Fig. 3 after titre (b) has attained an almost constant value suggests at first sight a continuance of polypeptide formation after amino-acid production has reached its maximum. Whether or not this be due to the activities of more than one enzyme it is difficult to state with certainty. In the gluten experiment with reaction conditions at pH 6 it is however probable that the proteinase and dipeptidase are active simultaneously, in which case a constant ratio between total titre and titre (b) cannot be expected. It is possible also for the dipeptidase to reach saturation point at a lower percentage of substrate than does the proteinase. In the case of edestin the reaction conditions $(p$H 4·1) would almost immediately cause the destruction of any dipeptidase.

It is therefore highly suggestive to discover that in this connexion there is no disturbance of the balance between polypeptide and amino-acid productions when edestin concentration is increased. There is an almost constant ratio between total titre and titre (b) (see Fig. 4), so that if produced the two curves should become horizontal at the same point. The activities of a single enzyme, namely, wheat proteinase, thus appear to account for the relevant facts.

The activation of wheat proteinase by cyanide using edestin as substrate has already been described [Mounfield, 1936, 2]. According to the hypothesis of Bersin [1933] and co-workers cyanide activates by reducing the S—S linkage of the inactive oxidized form of the enzyme to the SH form. This merely has the effect of increasing the quantity of active enzyme so that the course of any given proteolysis should not thereby be altered except in velocity. This reasoning is supported by the results of the experiments on the effect of cyanide on the relations between edestin concentration and initial reaction velocity (see Fig. 4).

The ratio total titre : titre (*b*) with cyanide is almost identical with the corresponding ratio without cyanide, namely, 2 : 1 in each case. This indicates quite clearly that cyanide caused no disturbance of the equilibrium between polypeptide and amino-acid production. The cyanide merely elevates both total titre and titre (*b*) curves to proportionate extents and does not induce a more complete hydrolysis of the split products.

The inability of cyanide to effect an increase in the rate of hydrolysis of gluten as measured by increase in total titre (see Fig. 3) is somewhat surprising since in most other respects this substrate behaves similarly to edestin. It is unusual to find that activation by cyanide depends upon the substrate. It is possible that the mechanism of attack by the enzyme is different in the case of gluten or it may be that the method of preparation of the substrate permits the introduction into the reaction mixture of reducing substances which completely activate the proteinase and render the cyanide ineffective.

Another explanation however presents itself. Since proteins most probably contain the S—S and SH type of linkages it is conceivable that they also are affected by cyanide in much the same manner as papain and related enzymes are considered to be affected. Such an assumption would explain not only the dependence of activation upon choice of substrate (gluten for example might be regarded as a fully "activated" protein, the addition of cyanide causing no further modification or increase in susceptibility) but it would also form the basis of a rational explanation of the extension of the range of specificity exhibited by cyanide-activated papain, cathepsin and yeast proteinase. According to Willstätter & Grassmann [1924] untreated papain attacks only gelatin, denatured albumin and possibly peptones whereas after activation native albumin, histones, protamines and even certain tripeptides are included within its range of substrates. An almost identical widening of substrate range occurs with cathepsin [Waldschmidt-Leitz *et al.* 1929] and yeast proteinase [Grassmann & Dyckerhoff, 1928]. Instead, therefore, of supposing that the enzyme is the activated component it is equally reasonable to consider the protein as being "activated" or rendered more susceptible to attack. Indeed, both enzyme and substrate may be affected in this way.

In spite of its inability to affect the increases in total titre in the gluten experiment cyanide does however cause a small but definite increase in titre (*b*) and a corresponding reduction in titre (*a*) (see Fig. 3). In accordance with the argument outlined above this disturbance in the equilibrium suggests that the quantity of amino-acid is augmented by a more complete hydrolysis of peptide linkages brought about in all probability by cyanide activation of the dipeptidase. That such is still possible to a small extent at *p*H 6 has already been demonstrated [Mounfield, 1936, 2]. Support is gained for this view from the data cited in Table I. In the absence of all substrate except that naturally present in the sprouted wheat extract by virtue of its method of preparation (probably wheat globulin and leucosin together with peptides etc.) slight activation by cyanide as measured by increase in total titre is again observed. Further, the same depression of titre (*a*) and increase in titre (*b*) is evident. The phenomenon is absent below about *p*H 5·5 but is prominent between *p*H 7 and 8 a range which covers the optimum for dipeptidase activity, to which the effect is no doubt due. The middle line in Fig. 3 is of course plotted from values obtained after deduction of such controls carried out in the presence of cyanide. Reference has already been made to the fact that precisely similar results were recorded during the measurement of the hydrolysis of glycylglycine and leucylglycine by wheat dipeptidase.

An unusual feature of this work has been the almost complete inability of the enzymes of sprouted wheat extract to attack either glutenin or gliadin as prepared by the methods outlined in the introduction to this paper. In the case of gliadin it is true there is evidence of a slight optimum at or near pH 5 but the extent of the hydrolysis in no way compares with that secured with other substrates. Doubling the concentration of substrate was without influence upon the titre increase, a fact which disposes of any error which might have arisen through the use of substrate concentrations below saturation point. The device of using a mixed substrate of glutenin and gliadin in equal proportions was also without avail—there appears to be no complementary relationship between the two proteins in this connexion.

It has been pointed out by Dakin & Dudley [1915] that contact with alkali, which causes racemization of proteins can render the protein molecule resistant to attack by proteolytic enzymes. This might conceivably be the explanation for the slight increase in the extent of hydrolysis of glutenin when prepared without contact with alkali, but it is certainly not responsible for the resistance shown by the gliadin used in these experiments.

Several possibilities suggest themselves but none appears to offer a satisfactory explanation of the phenomena. In the first place it is conceivable that by prolonged contact with reagents such as ethyl alcohol and acetic acid, necessary for their preparation, the proteins have been modified in such a way as to obstruct proteolysis. This however is contrary to commonly observed facts, since denaturation, coagulation and allied changes tend rather to render the protein structure more susceptible to attack. Secondly, the method of preparation may have introduced substances possessing power to inhibit proteolysis or, on the other hand, may have removed certain necessary activators. The latter two suggestions offer most promise, but so far work along these lines has proved unfruitful. Attempts to activate with cyanide, for example, have been unavailing.

SUMMARY

1. Wheat proteinase hydrolyses gelatin optimally at pH 5·1 and wheat gluten at pH 6·0 but does not attack ovalbumin, and affects glutenin and gliadin only slightly.

2. Amino-acid production from gluten reaches a maximum at about 1·5 % substrate concentration while half the maximum reaction velocity is reached at about 0·25 % gluten. Sodium chloride is without effect upon gluten hydrolysis.

3. The effect on initial reaction velocity at pH 4·1 of variation in edestin concentration is explicable on the assumption that only wheat proteinase is active. With gluten as substrate and reaction conditions at pH 6 the end products appear to be capable of further modification by the participation of wheat dipeptidase in the reaction.

4. Activation of wheat proteinase by cyanide using edestin as substrate merely affects reaction velocity and does not bring about a more complete hydrolysis of the cleavage products.

5. No activation with cyanide is observed when gluten is employed as the substrate for the proteinase. The decrease in the ratio titre (a) : titre (b) may be attributed to a slight activation of the concomitant dipeptidase.

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CCXVIII. PROTEINASE SECRETION AND GROWTH OF *CLOSTRIDIUM HISTOLYTICUM*

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YOUNG cultures of *B. fluorescens liquefaciens* quantitatively secrete a proteinase into the culture medium, as was found by Virtanen & Tarnanen [1931; 1932] and Virtanen & Suolahti [1937]. In previous work on the proteinase of *Cl. histolyticum* Weil & Kocholaty [1937] reached a similar conclusion.

Recently Maschmann [1938, 1] reported on a bacterial proteinase (*B. botulinus, B. Welchii*) which he considers to be in the "intracellularly acting proteinase of anaerobic bacteria", differing from the proteinase which is secreted by the bacterial cell. In a later publication on *Cl. histolyticum*, the same author [Maschmann, 1938, 2] concludes that this micro-organism produces three distinct proteinases. These are somewhat vaguely differentiated according to their actions on different protein substrates and their behaviour towards cysteine and other activators. Two of these enzymes are believed to be "extracellular" in character, being secreted in the early stages of bacterial growth and to be indifferent towards, or even inhibited by, cysteine and other common activators. The third enzyme is considered to be "intercellular", and to differ from the others in its ability to hydrolyse clupein but not gelatin, and to be activated by —SH compounds. Maschmann claims that the activation phenomena observed by us on culture filtrates of *Cl. histolyticum* are due to this intracellular proteinase, which is freed only by autolysis of the bacterial cell.

Our previous work on this micro-organism has been extended and the results are still at variance with the conclusions reached by Maschmann. In cultures of *Cl. histolyticum*, both the bacterial growth and the proteolytic activity (initial and full[1]) of the cell-free bacterial filtrates, reach a maximum value within 24 hr. after inoculation. This is true regardless of the type of medium employed provided that the latter has pH near the neutral point. In all cases here investigated it was possible to activate the proteinase by means of Fe^{++} in combination with sulphydryl compounds. Thus, a proteinase activated by Fe^{++}-cysteine was found in filtrates from 6 hr. cultures, at a time when practically no bacterial autolysis had yet taken place. Similar activation was observed in filtrates prepared after incubation periods up to 12 days: Fe^{++}-cysteine likewise activated the intracellular proteinase obtained by destroying the bacteria themselves with a supersonic oscillator. On the basis of the results there appears no justification for assuming a difference between the intracellular proteinase and the proteinase secreted into the culture medium.

Cell-free filtrates obtained after incubation periods ranging from 12 to 48 hr. were found to hydrolyse both clupein and gelatin. In every case activation by

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[1] By initial activity is meant the original proteinase activity without addition of activator; by full activity is meant the activity obtained in the presence of an Fe^{++}-SH activator. In the present work cysteine was used as the —SH source, underconditions described by Weil & Kocholaty [1937].

means of Fe^{++}-cysteine was observed. Maschmann states that the clupein-splitting enzyme is intracellular, and distinct from the gelatin-hydrolysing, extracellular enzymes. But its appearance in the culture filtrates (12 hr. incubation) before appreciable bacterial autolysis has occurred, indicates that it too is secreted and is probably identical with the gelatin-hydrolysing enzyme.

When cultures of *Cl. histolyticum* are incubated beyond the point at which maximum bacterial growth and proteolytic activity occur (24 hr.) a fairly rapid decrease in the activity of the proteinase takes place. This is not due to a temperature effect but appears to be related in some way to the autolytic processes. The stability of the bacterial proteinase depends also on the type of medium employed in the culture.

EXPERIMENTAL

The organism used was, as in previous work, a dissociated strain of *Cl. histolyticum* obtained from the American Type Culture Collection, No. 4872 [Hoogerheide, 1937]. The culturing was done as previously described, with the exception that the time of incubation was 24 hr. unless otherwise stated [Kocholaty & Hoogerheide, 1938]. The medium was in every case adjusted to pH 7·4 before inoculation. The estimation of proteolytic activity was carried out by measuring the liberation of free NH_2 groups from a gelatin substrate by the Van Slyke method. All results are expressed in terms of ml. 0·1 N base equiv. NH_2-groups liberated. Unless otherwise stated, the enzyme studies were made with cell-free solutions obtained by centrifuging and filtering the cultures through Seitz filters. Bacterial counts were made as previously described.

Secretion of proteinase

The relationship between bacterial growth and proteolytic activity is shown in Fig. 1. A horse meat broth medium (National Drug Co.) was inoculated with *Cl. histolyticum* and incubated at 37·5°. Samples were removed at intervals,
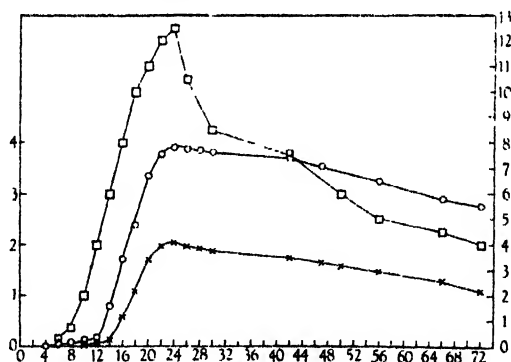
Fig. 1. Relation between growth and proteinase secretion of *Cl. histolyticum*. Abscissa: hr. bacterial growth after inoculation. Ordinate (left side): proteinase activity in ml. 0·1 N KOH. Ordinate (right side): no. of bacteria in 1 ml. × 10^{-8}. □ = no. of bacteria. ○ = full activity of *Cl. histolyticum* proteinase. × = initial activity of *Cl. histolyticum* proteinase.

filtered through Seitz filters and both initial and full activities measured. Bacterial counts were made at the same time. Both initial and full activities of the proteinase in the culture filtrates increased with increase in bacterial count up to a maximum at about 24 hr. After this a sharp drop occurred in the number of bacteria present and the initial and full proteolytic activities also decreased, but

at a slower rate. These results indicated the improbability of the proteinase being liberated into the medium as a result of bacterial autolysis. If such were the case an increase in the proteinase activity would be expected to occur after the 24 hr. maximum. Such an increase has never been observed. The proteinase which is activated by Fe^{++}-SH is already present in the filtrates 6 hr. after the inoculation, and increases rapidly with increase in bacterial count. This is doubtless an example of true bacterial enzyme secretion. In contrast to this finding, Maschmann claims that just this particular proteinase which can be activated by Fe^{++}-SH is the "intracellular proteinase, which is liberated only by autolytic processes occurring after the death of the bacteria, and which makes its appearance in the culture medium at a relatively late stage of incubation".

When the bacteria are separated by centrifuging and Seitz filtration from a culture which has not yet reached its maximal growth (15 hr.), it is found that the cell-free filtrate contains practically the whole of the proteolytic activity. The bacteria themselves attack gelatin only slightly.

Example: a 100 ml. portion of 3 % neopeptone, pH 7·4, was inoculated with *Cl. histolyticum*, and after 15 hr. incubation at 37·5° was passed through a Seitz filter. One ml. of the bacteria-free filtrate gave, in 20 hr. incubation with gelatin under the usual conditions, an initial activity of 1·56 and a full activity of 2·62 (ml. 0·1 N alkali). The entire mass of bacteria removed by centrifuging and filtration was washed, suspended in saline and subjected to the action of a supersonic oscillator until cell destruction was about 80 % complete. The proteolytic activity of the whole mass of broken-up bacteria, measured against gelatin as above was: initial, 0·26; full, 0·68. The proteolytic activity of the bacterial mass was only about 0·3 % of that of the whole filtrate. That the bacteria themselves do show a slight proteolytic activity may be due to the fact that they still contain small amounts of preformed, unsecreted proteinase. Since in a 15 hr. culture rapid cell proliferation is still taking place and little autolysis has occurred, the fact that almost the entire amount of proteinase is found in the filtrate is further evidence that a true secretion of enzyme as expressed both by initial and full activities has occurred. The bacteria themselves contain a polypeptidase and a dipeptidase which pass into the culture medium only on autolysis and which are not secreted by young cells under conditions in which the proteinase is secreted. This fact also supports the idea that the latter is liberated not by autolysis but by a truly secretory process.

In a second, similar experiment, 4 l. 2 % neopeptone were inoculated with *Cl. histolyticum* and incubated at 37·5° for 20 hr. The bacteria were collected by centrifuging, washed thoroughly with physiological saline and finally suspended in 10 ml. saline. 1 ml. of this suspension gave an initial activity against gelatin of 0·98 ml., and a full activity of 1·21 ml. When the bacterial suspension was first treated with the supersonic oscillator until 80 % cell destruction occurred, 0·2 ml. gave the following activities: initial 1·31 ml.; full, 3·29 ml. Also in this experiment the activation behaviour of the proteinase obtained from the bacteria themselves did not differ from that previously observed for the enzyme secreted into the culture medium.

If the changes occurring in the bacterial count and the proteolytic activity (both initial and full) in a *Cl. histolyticum* culture are determined from time to time one finds, as previously shown, that a maximum occurs at about 24 hr., after which a steady decrease takes place. If to such an incubation mixture in which the bacterial count has passed its maximum and is decreasing rapidly, one again adds fresh sterile culture medium, the bacterial count rises and reaches a new maximum at 24 hr. Simultaneously both the initial and full proteolytic

activities of the medium also increase to a new maximum. Further incubation beyond this point results again in a decrease in both bacterial count and proteolytic activity. A typical experiment is shown in Fig. 2. Enzyme activities were determined as before, on cell-free Seitz filtrates of the culture medium.
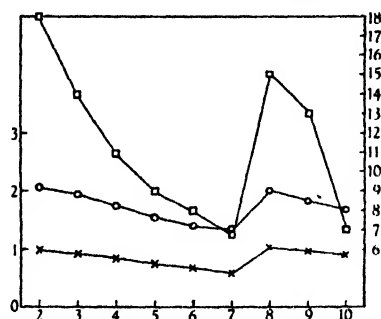
Fig. 2. Relation between bacterial growth and proteinase secretion. Abscissa: days of incubation. Ordinate (left side): proteinase activity in ml. 0·1 N KOH. Ordinate (right side): number of bacteria in 1 ml. × 10⁻⁵. □ =no. of bacteria. ○ = full activity of Cl. histolyticum proteinase. × = initial activity of Cl. histolyticum proteinase. Addition of new culture medium on the 7th day of bacterial growth.

Hydrolysis of clupein by the proteinase, and its activation by Fe^{++}-SH

When a bacteria-free culture filtrate of Cl. histolyticum (12 hr. growth) was allowed to act on gelatin or clupein, both substrates were split by the proteinase. In each case activation of the proteinase was obtained by addition of Fe^{++}-SH. Since in 12 hr. maximal bacterial growth has not nearly been reached (see Fig. 1) although clupein and gelatin are decomposed by the bacterial filtrate, this effect must be due to a proteinase which is secreted. After maximal growth has passed and autolysis of the bacteria occurs there is no increase in enzyme activity, either initial or full, towards clupein, as reported by Maschmann, but rather a decrease (Table I).

Table I. Hydrolysis of clupein by Cl. histolyticum proteinase

Cl. histolyticum grown in a solution of 3% heart infusion broth (Difco-standardized) at pH 7·4. After 12, 24 and 48 hr. growth at 37·5° samples were taken out and the Seitz-filtered enzyme solutions used for the exp. Determination: 2 ml. enzyme solution, 1 ml. cysteine-HCl (10 mg., neutralized), 0·6 ml. M/10 FeSO₄, or 1·6 ml. water, 2 ml. citrate-phosphate buffer pH 7·0 (McIlvaine) and 2 ml. 6·6% gelatin (neutralized) or 2 ml. of clupein sulphate (=50 mg. adjusted to pH 7·0). Digestion time was 5 hr. at 37·5°.

Age of bacterial culture (hr.)	Activity of the proteinase			
	Gelatin substrate		Clupein substrate	
	Initial	Full	Initial	Full
12	0·28	0·51	0·26	0·39
24	0·57	1·14	0·38	0·73
48	0·40	0·85	0·27	0·55

Since the activation behaviour, as well as the secretion, was found to be practically identical with respect to clupein and gelatin hydrolysis, there seems to be no reason to assume that the clupein-splitting enzyme differs from the one attacking gelatin, as Maschmann claims.

Stability of the proteinase

The decrease in both the initial and the full proteolytic activities after maximal bacterial growth has occurred is surprising. This decrease has been studied in three different culture media. It was found that the highest stability is shown in a casein medium. In neopeptone the stability is less, and about equal to that in horse meat broth (see Figs. 3 and 4). It seemed likely that the decrease
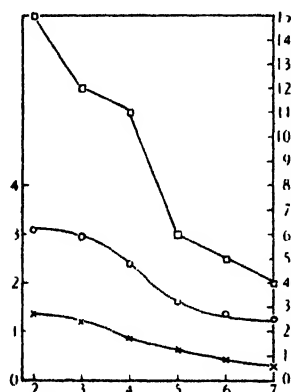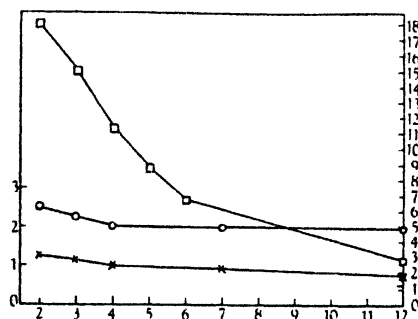
Fig. 3.   Fig. 4.

Fig. 3. Stability of *Cl. histolyticum* proteinase on neopeptone as culture medium. Abscissa: days of incubation. Ordinate (left side): proteinase activity in ml. $0.1 N$ KOH. Ordinate (right side): number of bacteria in 1 ml. $\times 10^{-5}$. ▢ — no. of bacteria. ○ — full activity of *Cl. histolyticum* proteinase. × — initial activity of *Cl. histolyticum* proteinase.

Fig. 4. Stability of *Cl. histolyticum* proteinase on casein as culture medium. Abscissa: days of incubation. Ordinate (left side): proteinase activity in ml. $0.1 N$ KOH. Ordinate (right side): no. of bacteria in 1 ml. $\times 10^{-5}$. ▢ = no. of bacteria. ○ = full activity of *Cl. histolyticum* proteinase. × = initial activity of *Cl. histolyticum* proteinase.

in the activity of the secreted proteinase in the culture medium might be due to inactivation during the long periods of incubation at 37.5°. Table II shows that this is not the case, since cell-free filtrates kept at 37.5° retain their proteolytic activity much longer than do unfiltered portions of the same incubation mixture. It is probable that the decrease is related to some process occurring during the autolysis of the bacteria.

Table II. Stability of Cl. histolyticum proteinase

Horse meat broth medium, 24 hr. culture; one portion (A) of the culture was allowed to stand at 37.5° without previous removal of the bacteria; a second portion (B) was freed from bacteria by Seitz filtration and also incubated at 37.5°; a third portion (C) was freed from bacteria and allowed to stand in the ice box. Enzyme determinations were made every 24 hr.

| | Proteinase activity | | | | | |
| | A | | B | | C | |
Days of incubation at 37·5°	Initial	Full	Initial	Full	Initial	Full
0	1·24	2·14	1·24	2·14	1·24	2·14
1	0·58	1·42	1·20	2·88	1·35	2·40
2	0·40	1·17	1·20	2·22	1·32	2·38
3	0·32	0·97	1·20	2·18	1·36	2·41
4	0·22	0·76	0·97	2·12	1·37	2·42

SUMMARY

The proteinase of *Cl. histolyticum* is liberated into the culture medium by secretion from the living bacteria, and is activated by Fe^{++}-cysteine under all conditions. On the basis of its activation behaviour, and its effect on various protein substrates, it appears to be identical with the proteinase obtained by breakdown of the bacterial cell.

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CCXIX. STUDIES ON THE ENDO-ENZYMES, PARTICULARLY THE PEPTIDASES, OF *CLOSTRIDIUM HISTOLYTICUM*

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THE first noteworthy work on bacterial peptidases as endo-enzymes was that of Virtanen & Tarnanen [1931; 1932, 1, 2] on *B. fluorescens liquefaciens*. Later, Gorini *et al.* [1932] and Gorbach & Pirch [1936–7] reported on the same subject. These various authors agree that the bacterial peptidases are present exclusively within the bacterial cell, are not secreted and show optimal activity at an alkaline reaction. Also in no instance has it been possible to demonstrate the presence of a carboxypeptidase in the bacterial cell.

In all of the above-mentioned work use was made of toluene autolysis to liberate the peptidases from the intact cell. Investigations carried out in this laboratory on the strict anaerobe, *Cl. histolyticum*, have shown that this time-consuming method of autolysis can be dispensed with to advantage by use of a supersonic oscillator to disrupt the bacterial cells. With this apparatus it is possible to break up the bacterial cells in a very short time, which is of particular advantage in view of the extreme instability of the bacterial peptidases in solution. When the tissue debris is removed from such preparation by filtration, a cell-free, water-clear fluid containing considerable quantities of peptidases is obtained. In this way it was shown that young cultures of *Cl. histolyticum* contain a polypeptidase with pH optimum at about 8·7, and a dipeptidase with an optimum at pH 7·6. All attempts to detect a carboxypeptidase failed, and it appears fairly certain that such an enzyme does not exist in bacteria. The bacterial polypeptidase is activated to a considerable extent by Mg^{++}, a phenomenon previously described by Johnson *et al.* [1936] for the animal polypeptidase, leucylpeptidase. The bacterial dipeptidase appears to be indifferent toward Mg^{++}.

Among the deamidases, we were especially interested in the enzyme which deaminizes *l*-aspartic acid. The deamination of this amino-acid by suspensions of *Cl. histolyticum* occurs optimally at pH 7·6 with formation of succinic acid as previously shown by Cook & Woolf [1928]. The presence of an aspartase could not be demonstrated, although previously this enzyme had been found in suspensions of *B. coli commune* by Quastel & Woolf [1926], and in cell-free preparations of *B. fluorescens liquefaciens* by Virtanen & Tarnanen [1932, 1, 2]. We were however able to show the presence of other deamidases, which in a weakly alkaline medium attack glycine, *l*-leucine, *d*-alanine, *d*-glutamic acid and *l*-tryptophan with liberation of NH_3.

In a previous publication Weil & Kocholaty [1937] suggested that the peptidases of *Cl. histolyticum* might be secreted by the intact cells. This idea was advanced because of the fact that no polypeptidase could be found in bacteria which had been autolysed with toluene. However, at that time determinations were made on very small quantities of bacteria and the activating effect of Mg^{++}

was unknown, so that the peptidase in question may have been present in the inactive form. The results now reported definitely indicate that these enzymes are not secreted, but must be classed as endo-enzymes.

EXPERIMENTAL

The micro-organism used, as in previous work, was the dissociated strain of *Cl. histolyticum* described by Hoogerheide [1937]. The culture medium used in all cases was a 2 % solution of neopeptone (Difco standardized), adjusted before inoculation to pH 7·4. After an incubation period of 18-20 hr. at 40°, the bacteria were centrifuged down, washed carefully, resuspended in saline and used at once. Such a suspension contains a negligibly small amount of spores and cellular debris, most of the bacteria being intact. The pH of the suspensions was always 7·0. When studies were to be made on cell-free material the washed bacteria were disrupted in the supersonic oscillator, after which the cellular debris was removed by Seitz filtration. The enzyme solutions obtained in this way were extremely unstable.

Under the conditions employed, a 10 min. treatment of a bacterial suspension in the supersonic oscillator resulted in a destruction of about 50 % of the cells. This could be increased to 80–85 % by a further treatment of 10 min. In order to avoid destruction of the enzymes the disruption was never carried beyond that point. This method for preparing bacterial enzyme solutions is advantageous not only because the cellular disruption is accomplished rapidly but also because it avoids the use of toluene which is sometimes harmful to enzymes. However, because of the formation of slimy material, bacterial suspensions treated in this way are very difficult to filter.

The estimation of peptidase activity was carried out by measuring the increase in COOH or NH_2 groups produced in the substrates leucylglycine and leucylglycylglycine, using respectively the method of Foreman [1920], or of Van Slyke. The estimation of NH_3 was by the usual distillation method.

pH optimum of the peptidases

To 1 ml. portions of the above described cell-free extracts were added 3 ml. citrate-phosphate buffer (McIlvaine—for the higher pH values KOH was also added); 5 ml. $M/5$ leucylglycine or 5 ml. $M/5$ leucylglycylglycine, adjusted to the proper pH, were added as substrates. The mixtures were incubated for 7 hr. at 40°. pH estimations made before and after the incubation period showed practically no change. The increase in free NH_2 groups was measured by the Van Slyke method, the results being expressed in ml. of 0·1 N base (Figs. 1 and 2). It should be pointed out that in all experiments reported the polypeptidase was accompanied by dipeptidase, since no attempt was made to separate the two enzymes. The values for polypeptidase activity are therefore only approximations.

Activation of the polypeptidase by Mg^{++}

Reaction mixtures were prepared consisting of 1 ml. cell-free enzyme solution prepared as described above, 5 ml. $M/5$ leucylglycine or leucylglycylglycine previously adjusted to the optimal pH, 3 ml. of citrate-phosphate buffer at the optimal pH and 1 ml. of water or $MgCl_2$ solution, the last to make the final molarity in the reaction mixtures 0·005–0·020. The mixtures were incubated at 40° for 7 hr., the COOH increase being measured by the method of Foreman. Results (Fig. 3) are expressed in ml. of 0·1 N KOH.
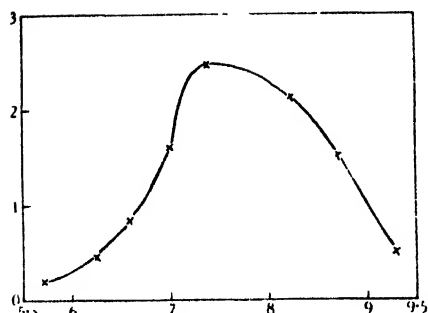
Fig. 1.
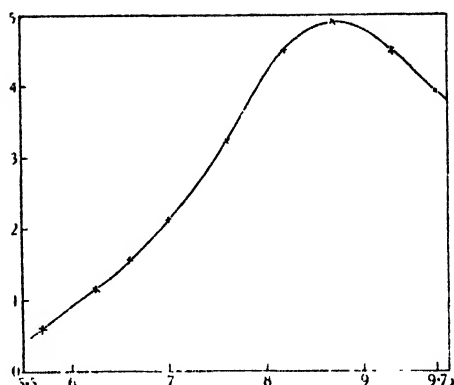
Fig. 2.

Fig. 1. pH optimum of *Cl. histolyticum* dipeptidase. Abscissa: pH. Ordinate: dipeptidase activity in ml. 0·1 *N* KOH.

Fig. 2. pH optimum of *Cl. histolyticum* polypeptidase. Abscissa: pH. Ordinate: polypeptidase activity in ml. 0·1 *N* KOH.

Fig. 3 shows the effect of conc. of Mg++ on the activity of *Cl. histolyticum* polypeptidase. Table 1 shows some results obtained with the bacterial dipeptidase at pH 7·6, and with the polypeptidase at pH 8·7. The Mg concentration was 0·01 *M* in the final reaction mixture. Comparative determinations were made using both a suspension of intact cells, and a cell-free enzyme solution prepared by the supersonic method.

Table 1. *Effect of Mg++ on the activity of* Cl. histolyticum *peptidases*

Enzyme source	Dipeptidase activity hr. incubation				Polypeptidase activity hr. incubation			
	1	4	7	24	1	4	7	24
Intact cells	—	—	—	0·91	—	—	—	1·24
Intact cells + Mg++	—	—	—	0·90	—	—	—	2·00
Disrupted cells	0·55	1·18	2·23	—	1·24	2·54	3·41	—
Disrupted cells + Mg++	—	1·15	—	—	2·04	—	3·76	—
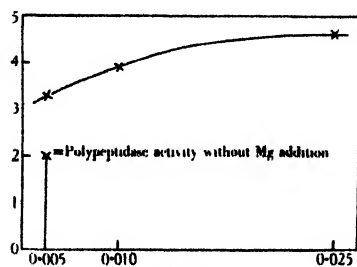
Fig. 3.
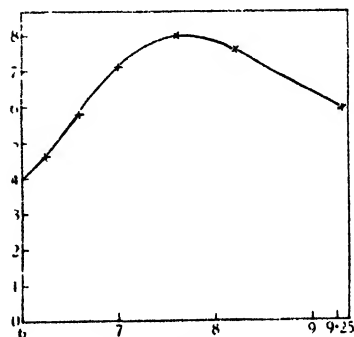
Fig. 4.

Fig. 3. Activation of *Cl. histolyticum* polypeptidase by Mg++. Abscissa: molarity of MgCl₂ added. Ordinate: polypeptidase activity in ml. 0·1 *N* KOH. χ : polypeptidase activity without Mg addition.

Fig. 4. pH optimum of *l*-aspartic acid deaminase (*Cl. histolyticum*). Abscissa: pH. Ordinate: ml. *N*/70 NH₃ liberated.

It is apparent that the bacterial dipeptidase is indifferent toward the presence of Mg^{++}. The extensive activation of the polypeptidase by Mg^{++} suggests that this enzyme might be largely inactivated by dialysis. Addition of Mg^{++} to such dialysed preparations should reactivate the enzyme, as was shown by Johnson et al. [1936] in the case of the intestinal leucylpeptidase. However, the polypeptidase preparations obtained were too unstable to test this point.

Cell-free Seitz filtrates, obtained from young actively-growing cultures (15 hr.) of Cl. histolyticum in which little autolysis has occurred, contain practically no dipeptidase or polypeptidase. Also from Table I it is seen that young washed intact suspensions of the bacteria show little peptidase action unless the incubations are carried out for long periods of time (24 hr.). The activity observed under such conditions is probably the result of enzyme liberation by autolysis, since toluene was present as a preservative during the long incubation period. But if the bacteria are previously broken up by the supersonic oscillator, high peptidase activity is observed even in short incubations. Such results indicate that these enzymes are endocellular, and are liberated only after destruction of the cell membrane by autolysis or other means.

Tests made under the same conditions for the presence of a bacterial carboxypeptidase, using chloroacetyl-l-tyrosine as substrate at pH 7·4 and 8·4, and with and without added Mg^{++}, were negative in each case.

Deamination of l-aspartic acid

For establishing the pH optimum of this enzyme, reaction mixtures were prepared consisting of 2 ml. bacterial suspensions (treated with the supersonic oscillator but not filtered), 2 ml. $M/10$ l-aspartic acid and 5 ml. citrate-phosphate buffer. The incubation period was 20 hr. at 40°. The results are shown in Fig. 4 in terms of ml. $N/70$ NH_3 formed. The pH optimum for the deamination was near 7·6.

Similar reaction mixtures, extending over the same pH range, but containing 2 ml. $M/10$ fumaric acid and 10·7 mg. NH_4Cl in place of the l-aspartic acid (total volume, 11 ml.) gave no evidence of aspartic acid synthesis after 20 hr. incubation at 40°. No appreciable decrease in free NH_3 occurred, indicating that the enzyme aspartase was absent.

In another experiment a fairly strong disrupted bacterial suspension was allowed to stand at pH 7·6 for 36 hr. with 4 ml. $M/10$ aspartic acid (40°). After filtration, 10 % H_3PO_4 was added, and the solution extracted several times with ether. The ether extract was evaporated to dryness, taken up in water, and treated with animal charcoal. On evaporation a crystalline material, M.P. 185°, was obtained. A mixed M.P. with pure succinic acid showed no depression. For further identification, the Pb salt was prepared, using Pb acetate. This salt was obtained in its characteristic form, small whetstone-shaped crystals arranged in rosettes. The deamination of aspartic acid yields succinic acid, as previously reported by Cook & Woolf [1928].

Deamination of other amino-acids

Because of the scarcity of enzyme material, it was not possible to determine the pH optima of the deamidases which attack other amino-acids. However, it could be shown that enzyme solutions obtained by the disruption method from Cl. histolyticum do bring about aerobic deamination of glycine, l-leucine, d-alanine, d-glutamic acid and l-tryptophan at pH 7·8. To 3 ml. enzyme solution

were added 2 ml. $M/10$ amino-acid solution and 5 ml. citrate phosphate buffer (McIlvaine). After 48 hr. incubation at $40°$, the NH_3 liberated was determined by the distillation method. The results are shown in Table II.

Table II. *Deamination of amino-acids by* Cl. histolyticum *enzymes*

Amino acid	ml. $N/70$ NH_3 formed after 48 hr.
Glycine	4·34
d-Alanine	3·68
l-Leucine	4·86
d-Glutamic acid	5·70
l-Tryptophan	2·14

Intracellular nature of the enzymes

All attempts to show the presence of the above enzymes (dipeptidase, aminopolypeptidase, deaminases) in cell-free filtrates obtained from young rapidly growing cultures were unsuccessful. In agreement with other authors it must be concluded that these enzymes are not secreted during bacterial growth, but are liberated only upon autolysis of the cells.

SUMMARY

1. Solutions obtained by disrupting *Cl. histolyticum* suspensions with a supersonic oscillator contain a dipeptidase with a pH optimum of 7·6, and a polypeptidase acting optimally at pH 8·7.

2. The polypeptidase is activated by Mg^{++}, while the dipeptidase is unaffected.

3. The preparations contain also enzymes which attack various amino-acids with liberation of NH_3.

4. All of the above enzymes are endo-enzymes, and are not secreted by the intact bacteria.

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CCXX. ENZYMIC ADAPTATION IN
CLOSTRIDIUM HISTOLYTICUM

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RECENTLY Kocholaty & Hoogerheide [1938] described an example of the adaptation of certain of the endo-enzymes of the strict anaerobe *Cl. sporogenes*: by varying the pH of the culture medium a considerable shift in the pH optimum of some of the dehydrogenases could be produced. It has now been found that under specified conditions the pH optimum of the proteinase secreted by this micro-organism can be shifted, and that, by repeated subculturings on gelatin or casein, specific proteinases are developed which will hydrolyse gelatin, but not casein, and *vice versa*.

EXPERIMENTAL

The methods used in preparing the *Cl. histolyticum* proteinase and estimating its activity were the same as previously described [Kocholaty *et al.* 1938]. Casein (Hammarsten) and gelatin (Coignet Gold Label) were used as culture media, and as substrates for proteinase activity determinations. Bacteria-free Seitz filtrates of the incubated cultures were used in the enzyme studies.

Shift in the pH optimum of the proteinase

The proteinase secreted by *Cl. histolyticum* grown at pH 7·2–7·4 on the common media, such as neopeptone, horse meat broth, gelatin or casein, has a pH optimum of 7·0 as found by Weil & Kocholaty [1937]. The same holds for the proteinase secreted by various other types of bacteria. When *Cl. histolyticum* is grown on casein medium at pH 6·0 for 20 hr. the secreted proteinase is no longer optimally active at pH 7·0 but at about pH 6·7. During the 20 hr. incubation, the pH of the culture medium increases from 6·0 to 6·7 or 6·8. If the culture medium
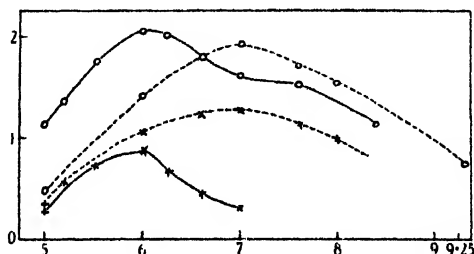
Fig. 1. pH optimum of *Cl. histolyticum* proteinase. Abscissa: pH. Ordinate: proteinase activity in ml. of 0·1 N KOH. ——— —in acid medium (3 % casein; 1 % glucose). ----- =in neutral medium (3 % casein). O =full activity. × =initial activity. Age of the culture: 20 hr.

is held at a definitely acid reaction during the whole 20 hr. of incubation a still larger shift in the pH optimum of the secreted proteinase takes place. A very simple method for achieving this effect is to add glucose to the culture medium. When *Cl. histolyticum* is grown on casein (3 %) in the presence of glucose (1 %),

extensive gas formation occurs as a result of sugar breakdown, and the pH of the culture medium drops, because of the formation of acids, from pH 7·4 to about 5·8 toward the end of the incubation period. The pH optimum of the proteinase found in filtrates from such cultures is now about 6·0. These facts are illustrated in Fig. 1. It will be seen that identical shifts occur in the pH optima both of the initial and the full (with cysteine plus Fe^{++}) proteinase activities.

This simple example of enzymic adaptability can be made still more striking. If a solution of 3 % casein at pH 7·4 is inoculated with a culture of *Cl. histolyticum* grown on casein plus glucose as described above, the proteinase secreted after 20 hr. growth is again found to be optimally active at pH 7·0 (both initial and full activities). It is evident that the nature of the proteinase secreted by the bacteria adjusts itself very easily and rapidly to changes in the culture medium.

The production of specific proteinases by Cl. histolyticum

The proteinase of *Cl. histolyticum* readily attacks casein, gelatin, horse meat broth, egg albumin, neopeptone and clupein among the media investigated. The hydrolysis, in every case, is activated by SH-heavy metal complexes, for example, by cysteine plus Fe^{++}. When the bacteria are grown on casein or gelatin the secreted proteinase attacks both casein and gelatin to about the same extent (see Table I, Exp. 1).

Among the easily available proteins of high mol. wt., casein and gelatin show the greatest divergence in their amino-acid compositions. For this reason these two proteins were chosen for the following experiments: the greater the difference in the proteins studied, the easier it should be to establish a specific enzyme adaptation in the proteinase secreted by *Cl. histolyticum* when repeatedly subcultured on the respective proteins. The main differences in the amino-acid compositions of casein and gelatin are shown below, the values being those given by Hawk & Bergeim [1931].

Amino-acid	% in gelatin	% in casein
Glycine	25·5	0·4
l-Hydroxyproline	14·1	0·2
d-Valine	0·0	7·9
d-Hydroxyglutamic acid	0·0	10·5
l-Tyrosine	0·01	6·5
l-Tryptophan	0·0	2·2

By repeated transfers of *Cl. histolyticum* cultures from gelatin to gelatin, and from casein to casein medium, an attempt was made to develop specificity in the secreted proteinase for one or the other of these proteins. The transplantations were made as follows: inoculations of *Cl. histolyticum* from the stock culture were made in test tubes containing 10 ml. 10 % gelatin or 10 % casein. After bacterial growth had become well established,[1] 1 ml. portions of these incubation mixtures were transferred into fresh 10 % gelatin and casein media, respectively. This operation was repeated 6 times, always transferring from gelatin to gelatin, and from casein to casein. The incubation period during these

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[1] It was frequently found that *Cl. histolyticum* did not grow readily on gelatin medium. When ordinary gelatin was used an entire week often elapsed before bacterial growth began. This induction period could however be shortened by using a gelatin medium which had first been partially digested by trypsin, and then sterilized. Usually after one subculture on such trypsin-digested gelatin, the bacteria became sufficiently adapted so that satisfactory growth and enzyme secretion could be achieved on ordinary (not previously decomposed) gelatin. With casein, on the other hand, good growth and enzyme secretion were usually obtained after the first inoculation, no extensive induction period occurring.

sub-culturings varied from 24 to 48 hr., satisfactory bacterial growth occurring within this time. After the 6th transfer, larger amounts of 3% gelatin and 3% casein were inoculated with the respective cultures, and incubated for 24 hr. The solutions obtained after removal of the bacteria by Seitz-filtration were used in the enzyme studies.

Table I. *Formation of specific proteinases by* Cl. histolyticum

Enzyme ...	G		G		C		C	
Substrate ...	Gelatin		Casein		Gelatin		Casein	
1 Initial activity	1·47		1·36		1·20		1·12	
Full activity	2·51		2·69		2·34		2·43	
		*		*		**		**
2 Initial activity	1·18	1·12	0·38	0·54	0·46	0·70	1·00	0·97
Full activity	2·16	2·00	1·88	1·81	1·80	1·79	2·27	2·24
3 Initial activity	1·28	1·20	0·00	0·40	0·00	0·37	1·18	1·12
Full activity	2·50	2·68	1·20	1·28	1·47	2·00	2·32	2·41
4 Initial activity	0·30		0·18		0·24		0·41	
Full activity	0·59		0·36		0·46		0·89	

Explanation of Table I:

G = Enzyme solution obtained by growing *Cl. histolyticum* on gelatin medium.

C = Enzyme solution obtained by growing *Cl. histolyticum* on casein medium.

Activity determinations were made on cell-free bacterial filtrates using gelatin and casein as substrates. Reaction mixtures contained 2 ml. filtrate, 3 ml. 6·6% casein or gelatin adjusted to pH 7·0, 5 ml. citrate-phosphate buffer (McIlvaine) at pH 7·0 and 1·6 ml. water (for initial activity) or 1 ml. neutralized cysteine-HCl (10 mg.) plus 0·6 ml. $N/10$ FeSO$_4$ (for full activity). Incubation time was 20 hr. at 40°. Estimations were made by the Van Slyke method, results being expressed in ml. 0·1 N base equiv. to the $-NH_2$ groups liberated.

Exp. 1. Comparison of proteinases obtained in casein and gelatin media after one inoculation. The bacterial filtrate was prepared from a culture of *Cl. histolyticum* grown for 24 hr. at pH 7·4 on 3% gelatin or casein.

Exp. 2. Specificity of proteinases obtained after 6 transplantations on 10% casein and 10% gelatin.

Column * = addition of 15 mg. *d*-valine, 13 mg. *l*-tyrosine, 4·3 mg. *l*-tryptophan and 21 mg. *d*-hydroxyglutamic acid to 10 ml. enzyme solution before activity determination.

Column ** = Addition of 50 mg. glycine and 28 mg. *l*-hydroxyproline to 10 ml. of enzyme solution before activity determination.

Exp. 3. Same as Exp. 2 except that 12 successive transplantations were made.

Exp. 4. Loss of specificity of *Cl. histolyticum* proteinases. Casein-trained bacteria (12 transplantations) were grown for 24 hr. on 100 ml. 3% gelatin supplemented with 150 mg. *d*-valine, 130 mg. *l*-tyrosine, 43 mg. *l*-tryptophan, and 210 mg. *d*-hydroxyglutamic acid. Gelatin-trained bacteria (12 transplantations) were grown for 24 hr. on 100 ml. 3% casein supplemented with 500 mg. glycine and 280 mg. *l*-hydroxyproline. After Seitz filtration, the enzyme solutions thus obtained (designated C and G, respectively) were tested for activity as described above.

As shown in Table I, Exp. 2, small differences are evident, after 6 transplantations, in the specificities toward gelatin and casein exhibited by enzymes developed respectively in gelatin and casein media. Particularly with reference to the initial activity, the gelatin-trained enzyme has lost some of its ability to hydrolyse casein, while the casein-trained enzyme attacks gelatin less readily than it does casein. By adding to the gelatin and casein enzymes, respectively, the more important amino-acids missing in casein and gelatin a slight increase in the initial proteinase activity towards these substrates is observed.

The transplantation of the above gelatin and casein cultures was continued as before, from gelatin to gelatin, and from casein to casein, until a total of 12 sub-culturings had been made. Then larger amounts of 3% gelatin and 3% casein media were inoculated, incubated for 24 hr., and the bacteria removed by Seitz filtration. Enzyme studies on the cell-free filtrates gave the results shown in Table I, Exp. 3. In the absence of added activator, the gelatin-trained enzyme

is no longer able to hydrolyse casein, and the casein-trained enzyme does not attack gelatin. Rather remarkable is the fact that addition of the activator, cysteine-Fe^{++}, reduces this specificity, so that the gelatin-enzyme now hydrolyses casein, and vice versa, the casein-enzyme attacks gelatin. Interesting also is the confirmation of the observation reported in Table 1, Exp. 2, that the gelatin-enzyme can be somewhat activated towards casein by addition of the amino-acids missing from the latter, but present in gelatin. The same holds, *mutatis mutandis*, for the casein-trained enzyme.

When casein is inoculated with *Cl. histolyticum* which has been carried through 12 sub-culturings on gelatin, no bacterial growth takes place even in 2 weeks of careful incubation. The same holds true when gelatin is inoculated with the casein-trained bacteria. However, when the culture medium consists of gelatin plus those amino-acids which are missing from this protein but present in gelatin, the casein-trained bacteria will develop a fair growth in several days. Likewise the gelatin-trained bacteria will slowly grow on a casein medium, supplemented by the amino-acids absent from the latter but present in gelatin. As shown in Table I, Exp. 4, the enzymes secreted in these latter cases are able to attack both casein and gelatin again, although not very strongly.

DISCUSSION

For the sake of brevity, the specific enzyme obtained by repeated sub-culturings on gelatin is termed "gelatinase", and that obtained in casein medium, "caseinase".

The results just described are of considerable interest from several points of view. By systematically training *Cl. histolyticum* on media such as casein or gelatin it is possible to develop two distinct proteinases of such extraordinary specificity that they can sharply differentiate between casein and gelatin. Also by simply culturing the casein-trained bacteria on a gelatin medium supplemented by those amino-acids missing from gelatin but present in casein, an enzyme is produced which again attacks both proteins. The same result is achieved by growing the gelatin-trained bacteria on casein supplemented by amino-acids missing from this protein but present in gelatin. Such results indicate that the specificity of these proteinases must be of very labile quality, depending only on the nature of the proteins used in their production. Addition of simple amino-acids to the culture medium, in fact, suffices to cause the specificity differences to disappear. The results presented in Table I, Exp. 4, show how easily these "trained" bacteria, and their secreted proteinases, can revert to their original state.

Of particular interest is the behaviour of the specific proteinases toward cysteine plus Fe^{++}. In the absence of this activator, the "gelatinase" and the "caseinase" fail to attack casein and gelatin, respectively, but both substrates are hydrolysed by each of these enzymes when the activator is present. There are two possible explanations for this SH-Fe^{++} effect.

1. The initial activity of the proteinase undergoes an activation in the sense that an extension of the field of specificity of the enzyme occurs. In other words, SH-Fe^{++}-activation broadens the specificity range so that different types of proteins can be attacked.

2. A 2-enzyme system may be present, as once postulated for papain by Bergmann [1937]. In this case the initial activity would represent a proteinase I, which would not be influenced by SH-Fe^{++}. A second enzyme, proteinase II, would be inactive under normal conditions, and active only in the presence of

heavy metal-SH complexes, and could be perhaps a type of peptidase. Attempts are being made to decide between the two ideas. Preliminary results indicate that the first theory is probably correct.

Recently two reviews of previous work on the subject of enzymic adaptation in micro-organisms were presented by Yudkin [1938] and Karstroem [1938]. Both authors distinguish between "adaptive" and "constitutive" enzymes. Quastel [1937] holds the view that the so-called constitutive and adaptive enzymes do not represent entirely different classes of enzymes. They represent rather the limits of variability of enzymes in a cell, the constitutive having the least range, and the adaptive the greatest range, of variability under different environmental and nutritional conditions. Our own view goes still further: we are inclined to picture the differentiation between constitutive and adaptive enzymes as a function of time; the longer a micro-organism is trained under altered environmental or nutritional conditions, the more will the properties of the constitutive enzymes disappear, until finally complete adaptation occurs. The many examples of the dissociation of various bacterial strains indicate that this view is probably not far wrong.

Yudkin [1938] has developed an interesting "mass action theory" of enzyme formation in bacteria. He postulates the existence of various inactive enzyme-precursors in the bacterial cell, and explains enzymic adaptation not by the formation of new enzyme, but rather by the conversion of the already existing precursor into active enzyme. The addition of substrate results in shifting the equilibrium of the enzyme-precursor system in the direction of active enzyme formation. On this basis, the results presented in Fig. 1, on the shift in the pH optimum of the secreted proteinases, would have to be explained as follows: the cultivation of $Cl. histolyticum$ on gelatin at a low pH results in the secretion of a proteinase, already present in the cell in the precursor form, which attacks gelatin optimally at an acid reaction; cultivation in neutral gelatin results in the secretion of a different enzyme, also present in inactive form, which splits gelatin optimally at neutral reaction. Such an assumption is difficult to justify. The only difference in the stimulations to which the bacterial cells are subjected in the two cases is the difference in the pH of the media; the substrate remains the same. A more reasonable theory appears to be that recently proposed by Quastel [1937] which treats the enzymes as "cell metabolites whose rates of formation and destruction follow the same physicochemical laws as those controlling the metabolism of any other metabolite of the cell".

The action of an enzyme depends essentially on its active group. On the basis of known facts, it may be tentatively suggested that perhaps not every enzyme present in the bacterial cell has its own characteristic colloidal carrier. It may be that only a limited number of different colloidal carriers are present, which are interchanged as necessity demands, and according to definite laws, among the active enzyme groups already present or formed during bacterial growth. The extent of substrate specificity can of course be influenced also by the more detailed structural characteristics of the colloidal carrier. The views of Quastel and Yudkin may perhaps be brought into harmony as follows: the "enzyme-precursor" postulated by Yudkin may be identical with the colloidal carrier which is in equilibrium with the various prosthetic groups; through various external influences these equilibria within the cell are disturbed, so that on the basis of the mass action law a coalition between colloidal carrier and active groups occurs, resulting in the formation of a new enzyme.

Summary

Several examples of enzymic adaptation in the proteinase of *Cl. histolyticum* are described. By changing the pH of the culture medium it is possible to change the pH optimum of the secreted proteinase. By repeatedly subculturing the bacteria on gelatin or casein media, enzymes are finally obtained which hydrolyse casein but not gelatin and vice versa. The mechanism of bacterial enzyme formation is discussed.

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CCXXI. THE PREPARATION AND PROPERTIES OF A HIGHLY ACTIVE CATALASE FROM HORSE LIVER

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THE enzyme catalase has been investigated by a great number of workers. Zeile & Hellström's [1930] method of preparing horse liver catalase has been used with some slight modifications by most investigators. The most active preparation so far obtained is that of v. Euler & Josephson [1927] (Kat. f. 43,000). Sumner & Dounce [1937] have recently described a different method for the preparation of catalase from beef liver. Sumner's crystalline catalase showed the activity 28,000.[1] The present paper reports the preparation of an enzyme from horse liver, with an activity of 55,000–60,000.

The method of preparation

2 kg. horse liver are ground and extracted twice with 2 l. water for 6 hr. Haemoglobin and other inert proteins are precipitated with alcohol and chloroform according to Tsuchihashi [1923]. After centrifuging, the clear supernatant solution is evaporated at a low temperature down to 2 l. and mixed with 3 l. saturated ammonium sulphate solution. The precipitate is dissolved in 1·5 l. water after which an equal vol. of 95 % alcohol is added. Precipitated protein and salts are centrifuged down and 700 ml. alcohol more are added. The enzyme is precipitated and is separated from the solution after which it is dissolved in 1 l. water. 800 ml. saturated ammonium sulphate are added again. A compound with a high Fe-content (about 16 %) is thrown down. This compound accompanies the catalase, when preparations are made according to previously described adsorption methods, and is the cause of the widely different values reported for the iron content. This iron is easily split off and after acid hydrolysis gives the characteristic red colour with NH_4CNS. The ammonium sulphate concentration is raised to 58–60 % and the enzyme precipitate is filtered off. It is dissolved in water, reprecipitated with alcohol, and the preparation is then dialysed. The activity is 40,000–43,000. The preparation is stable for months at 0°. The yield is 0·8 g.

The preparation is further purified by adsorption on tricalcium phosphate. The diluted solution of enzyme (0·2 mg./ml.) is adjusted to about pH 5·5 by addition of KH_2PO_4 and is then filtered through a column of tricalcium phosphate as is usual with chromatographic analysis. The enzyme is adsorbed in a distinct layer at the top of the column and then eluted by a phosphate buffer at pH 8. The catalase solution is dialysed in cellophane bags.

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[1] During Prof. Sumner's stay in Uppsala last winter I made a joint determination with him of Kat. f. of catalase, in the course of which we confirmed that our procedures were identical. The present values of Kat. f. are therefore directly comparable with those of Sumner & Dounce.

The activity of the preparation is 55,000–60,000. This procedure has been repeated a number of times with the same result.

The analyses show that the preparation contains 15·5 % N (by micro-Kjeldahl) $0·085 \pm 0·005$ % Fe (by the method of Jorpes), and 0·02–0·03 % Cu [by the [1933] method of Eisler et al. 1936].

For the determination of the activity the method described by v. Euler & Josephson [1927] was used. Kat. f. $= \dfrac{\text{reaction constant}}{\text{weight of preparation in g.}}$.

Determination of the spectrum

The light absorption at different wave-lengths was estimated by a photo-electric apparatus like that described by Warburg & Negelein [1929].

Stern [1937] has recently reported a quantitative determination of a catalase spectrum in the visible range. His preparation does not seem, however, to have been highly purified.
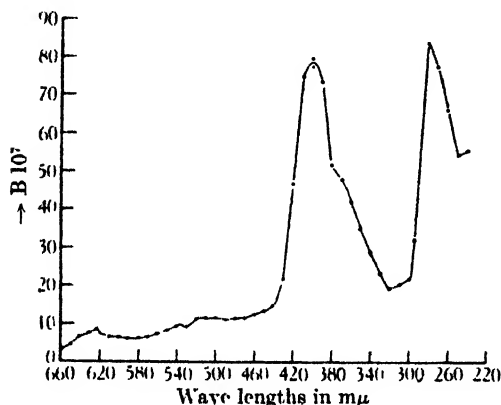
Fig. 1.
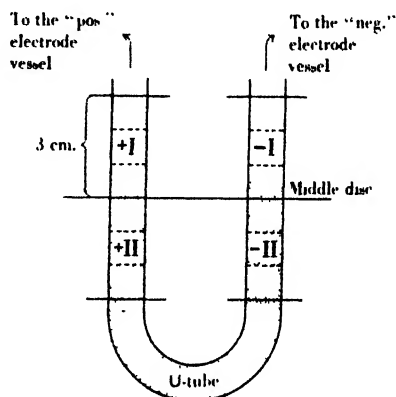
Fig. 2. The approximate position of the coloured preparation after the cataphoresis.

The molecular absorption coefficients are calculated from $\beta = \dfrac{1}{d}\dfrac{1}{c} \ln \dfrac{I_0}{I}$.

$d =$ the depth of the cell in cm.

$$c = \frac{\text{g. preparation}}{\text{ml.} \times \text{mol. weight}}.$$

I_0 represents the intensity of the incident light and I the intensity of the transmitted light.

Table I. *The molar coefficients at the maxima of absorption*

Wave-length mμ	Molar absorption coefficients
623	$8·3 \times 10^7$
536	$9·4 \times 10^7$
400	$78·5 \times 10^7$
280	$85·0 \times 10^7$

Cataphoresis experiments

The purity of the preparation was tested with the cataphoresis apparatus of Theorell [1934]. The boundaries between the catalase solution and the buffer solution were level with the middle disk at the start of the experiment. During

the experiment the preparation migrated towards the anode. The displacement was counteracted by transferring the liquid from one vessel to the other, according to the principle introduced by Tiselius [1937]. In this way it was possible to have the boundaries of the enzyme solution at the end of the experiment approximately in the same position as at the beginning.

Conditions: pH 6·99. Conductivity $1·37 \times 10^{-3}$. Current 5 mA. Time: 22 hr. and 40 min. Temp. 20°

8×2 ml. were brought from the "negative" electrode vessel to the "positive", corresponding to a displacement of the free boundaries of 8 cm.

The results of the analyses from the experiments are given in Table II.

Table II

		mg. prep. ml.	Kat. f.	% N	$\dfrac{10^{-2}k/\text{ml.}}{\text{N mg.}}$ ml.	% Fe	$\dfrac{10^{-1}k \text{ ml.}}{\text{Fe } \mu g.}$ ml.	% Cu	$\dfrac{10^{-2}k/\text{ml.}}{\text{Cu } \mu g./}$ ml.
The solution contained in the three cells above the middle disk	+I	2·0	50,000	14·9	3·30	0·1	7·8	—	—
Ditto below the middle disk	−I	5·0	51,000	15·5	3·47	0·082	6·6	0·044	1·15
,,	+II	9·55	54,000	15·8	3·35	0·091	5·8	0·027	1·9
,,	−II	11·4	52,000	15·3	3·41	0·079	6·2	0·026	2·0
The preparation in the U-tube		11·5	51,000	15·9	3·21	0·081	6·3	0·026	1·7
Preparation not submitted to cataphoresis		12·5	56,000	15·7	3·62	0·082	6·8	0·023	2·4

The contents of nitrogen and iron were both proportional to the activity of the solution in the cells. The copper also accompanied the activity, but in one cell (−I) the percentage was double as much as that usually found.

Ultracentrifuge experiments

Thanks are due to Prof. Svedberg for the opportunity of performing ultracentrifuge experiments at his Institute in co-operation with Nils Gralén. Using the light absorption method and light of wave-length 546 mμ the sedimentation constant of the iron-porphyrin-protein was found to be $11·2 \times 10^{-13}$. The preparation proved however not to be homogeneous. Ultraviolet light photographs, 250–290 mμ, showed the presence of a component with a sedimentation constant of about $3·2 \times 10^{-13}$. From dry weight determinations of the content in the two compartments of a "separation cell" [Tiselius et al. 1937] the concentration of this component is calculated to be 15–20 % of the total protein.

The diffusion constant was measured by the method of Lamm & Polson [1936] and was found to be $4·3 \times 10^{-7}$ while the partial specific volume was determined as 0·715. Using the formula $M = \dfrac{RT\,s_{20}}{D_{20}(1 - V\zeta)}$ the molecular weight is calculated to be 225,000.

Stern & Wyckoff [1938] found the value 11×10^{-13} for the sedimentation constant of horse liver catalase.

Sumner & Gralén [1938] have found the sedimentation constant[1] for crystalline catalase from beef liver to be $11·3 \times 10^{-13}$ and the diffusion constant $4·1 \times 10^{-7}$, which give a value of $M = 248,000$.

Separation of a copper-protein from the iron-porphyrin-protein

The preparation (2 mg./ml. in $M/15$ Na$_2$HPO$_4$) was mixed with 1/3 of its volume of a saturated solution of picric acid. Then $N/10$ acetic acid was added till a precipitate appeared at pH 5·2. This was centrifuged down and more

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[1] Corrected values, personal communication.

acetic acid added to the solution to bring the pH to 4·6, at which another fraction precipitated. These two fractions were dissolved separately in $M/15$ Na_2HPO_4 and were dialysed free from picric acid.

Each fraction has separately a considerably lower activity than the preparation before separation, i.e. fraction 1: Kat. f. 6000 and fraction 2: Kat. f. 3500. If however the original preparation is mixed with an equal volume of picric acid and the precipitation carried out by the addition of enough acetic acid to precipitate both fractions together at pH 4·6, the preparation retains the greater part of its activity after removal of the picric acid. This makes it probable that the inactivation is not due to the slightly acid reaction or to the action of the picric acid. It is possible therefore that both components may be necessary for the catalase activity.

The fraction which is first precipitated has an Fe content about 0·1 % and almost no Cu, whilst the other fraction has a Cu content of 0·16 % and very little Fe.

The sedimentation constants of the two isolated components have been determined. The Fe-porphyrin-protein had a sedimentation constant of about 12×10^{-13} and that of the Cu-protein was $3·27 \times 10^{-13}$.

It is probable that the preparation by picric acid precipitation gives rise to the same two fractions which were observed in the earlier ultracentrifuge experiments, mentioned above.

Kubowitz [1937] has recently shown that an oxidative enzyme, polyphenoloxidase, is a Cu-protein (0·165 %). Keilin & Hartree [1938] have shown that the Fe in catalase is reduced by hydrogen peroxide from the trivalent to the bivalent state. It is however immediately oxidized in the presence of molecular oxygen. It seems possible, that the Cu-protein in this highly active catalase preparation might—by analogy with Kubowitz' oxidative enzyme—be necessary for the re-oxidation of the bivalent Fe of the reduced catalase. The Cu-protein would in such a case be an integral part of a catalase system.

The activity was not, however, restored by a simple mixing of the two fractions nor was it raised by the addition of the Cu-protein to the original preparation. Further studies will however show, whether it is possible to separate the original preparation into two inactive fractions by other means than addition of picric acid and whether, in such a case the activity will be restored after mixing.

The differences in the activity of the preparation of Sumner & Dounce and that here described may be due not to impurities but to a difference in the structure of the protein (the preparations are made from different species of animal) or, if the Cu-protein is significant in the activity, to a different content of this substance. No information is given by Sumner & Dounce concerning the presence of copper.

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CCXXII. THE DISINTEGRATION OF TOBACCO MOSAIC VIRUS PREPARATIONS WITH SODIUM DODECYL SULPHATE

By MOTNAHALLI SREENIVASAYA AND NORMAN WINGATE PIRIE

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IT is well known that many bacteria are lysed and that toxins and antigens are modified by treatment with surface-active materials such as soaps and bile salts. Recently the commercially available wetting and spreading agents have been used in this way. Dean has summarized the chemical nature of these substances [1937]. The inactivation of diphtheria toxin by lauryl sulphate and the lysis of Gram-negative organisms have been studied by Bayliss [1936, 1937], and other workers, continuing the work of Larson *et al.* [1925], have prepared bacterial antigens by incubating suspensions of bacteria with these substances. This technique probably has no general application, for we have found that many large molecules are destroyed under these conditions. The destruction of preparations of potato virus " X " and of bushy stunt virus by incubation with the sodium salt of the sulphuric half ester of dodecyl alcohol has already been described [Bawden & Pirie, 1937; 1938, 1], and in this paper some observations on the disintegration of tobacco mosaic virus preparations will be reported.

Of this group of substances sodium dodecyl sulphate (Imperial Chemical Industries) and the apparently similar product called "Sulphonated" Lorol (Rönsheim and Moore) are the most efficient virus-inactivating agents that have been tested but the corresponding esters of cetyl alcohol and of the alcohols derived from palm oil, and the related amide, Igepon T, have some action; only sodium dodecyl sulphate (called SDS in future) has been studied in any detail.

The rate of action depends on temperature, concentration of SDS and pH. When tobacco mosaic virus preparations are disintegrated by SDS they become more acid, as they do when boiled [Bawden & Pirie, 1937]; it is therefore necessary to add a buffer to the system. Buffer solutions containing phthalates, phosphates or borates cannot be used, for even when dilute these form insoluble crystalline complexes with SDS. These complexes have not been investigated further. Veronal does not apparently combine with SDS and this buffer system [Michaelis, 1930] has been used. The action proceeds very slowly if at all in fluids more acid than pH 7·0: at pH 8·0 disintegration takes place and this pH value has been used in most experiments for in more alkaline solutions, although the action proceeds faster, there is greater spontaneous breakdown of the virus preparation.

The course of the action may be followed in five different ways, for on disintegration tobacco mosaic preparations lose their infectivity, serological activity, anisotropy of flow and sedimentability in a high-speed centrifuge and phosphorus and carbohydrate are no longer found in the precipitate which separates when the solution is one-third saturated with ammonium sulphate. An experiment in which the loss of infectivity and serological activity of a potato virus " X " preparation were followed has already been published [Bawden & Pirie, 1938, 1] and substantially similar results have been obtained with tobacco mosaic virus.

In this work a good preparation of virus, made by the method already described [Bawden & Pirie, 1937], was used. This preparation was colourless and a 2 % neutral solution was nearly clear and wholly in the liquid crystalline state; it gave specific precipitation with antisera at a dilution of 1 : 8,000,000 and contained 0·5 % phosphorus and 2·5 % carbohydrate. The protein shows strong anisotropy of flow when a 1 % solution is made in $M/20$ veronal buffer at pH 8·0; this solution gives an immediate precipitate when mixed with an equal volume of a 1 % solution of SDS in buffer. The precipitate has the sheen and "crystalline", or more correctly fibrous [Bernal & Fankuchen, 1937], appearance under the microscope that characterizes the precipitates given by tobacco mosaic virus with acids, strong salt solutions or clupein. After about $\frac{1}{2}$ hr. at room temperature or after a shorter time at 37° the precipitate dissolves and the anisotropy of flow can now be followed by shaking the mixture in a tube about 1 cm. wide between crossed Nicols. The intensity of the anisotropy of flow falls off rapidly and after about 2 hr. at 37° it is barely perceptible, i.e. it corresponds to that given by a 0·04 % solution of the original preparation. The anisotropy of flow disappears completely after about 10 hr. incubation. Nothing can be sedimented from a solution treated in this way by centrifuging for 3 hr. at 14,000 r.p.m. (17,000 times gravity). If the incubation is stopped before the anisotropy of flow has disappeared completely a small birefringent pellet with the properties of tobacco mosaic virus is sedimented and material can be isolated, which contains carbohydrate and phosphorus and which precipitates specifically with tobacco mosaic antisera.

If samples of a buffered mixture containing 0·5 % of both virus preparation and SDS are withdrawn after various periods of incubation and added to 2 vol. of $\frac{1}{2}$ saturated ammonium sulphate solution containing enough ammonia to make the solution alkaline to phenol red, it is found that the amounts of carbohydrate and phosphorus in the supernatant fluid after centrifuging increase as the anisotropy of flow disappears and that the ammonium sulphate precipitate is free from nucleic acid when it separates from a solution giving no anisotropy of flow. This shows that in addition to inactivating the virus and breaking down the protein of high molecular weight the SDS separates the nucleic acid from the protein to which it was attached.

The action, as measured by any of these methods, is very greatly affected by changes in the concentration of SDS and in the ratio of its concentration to that of the virus preparation. If the concentration of SDS is doubled the disruption of the virus is complete in 1 hr. at 37°, whereas if the concentration is halved the anisotropy of flow does not disappear completely even after many days if the initial virus concentration is 0·5 %, but if the virus concentration is reduced to 0·2 % the anisotropy of flow disappears in the usual way.

To prepare a quantity of the nucleic acid-free protein 50 ml. of a 1 % solution of virus preparation are mixed with 15 ml. of 5 % SDS. It is unnecessary to buffer the mixture but $N/10$ NaOH is added from time to time to keep the pH between 8·0 and 8·5. After incubation for 24 hr. at 37° the mixture is filtered on a grade 4·5 Bechhold membrane (Schleicher & Schull). Most, but by no means all, of the SDS is found in the filtrate. The filter residue is dissolved in 5–10 ml. of water and dialysed thoroughly in a cellophane tube; this is necessary for the SDS is easily precipitated by ammonium sulphate. After a few days the dialysis sac contents no longer froth strongly. The pH of the fluid is now adjusted to 8–9 and saturated ammonium sulphate solution is added; with about 1/10 of a volume there is copious precipitation and on centrifuging the precipitate separates as a clear, gelatinous, coherent mass; this is redissolved and repreci-

pitated in the same way. If the disruption of the virus preparation has been complete there will be no further precipitation on the addition of more ammonium sulphate but if any protein remains undisrupted it will be precipitated. Nucleic acid precipitates from the supernatant fluid on acidification; if however the ammonium sulphate precipitation is carried out under slightly acid conditions part of this nucleic acid will precipitate with the protein.

The nucleic acid-free protein may be precipitated many times with ammonium sulphate but it is found that this treatment converts it in part into an insoluble material and in part into protein that is very easily precipitated by ammonium sulphate. After four or five reprecipitations it precipitates with 1–2 % solutions of ammonium sulphate and of certain other salts. This apparent denaturation also takes place if the neutral protein is kept at room temperature for some days and even more rapidly if it is made slightly acid. The insoluble protein, like the denatured protein made from tobacco mosaic virus preparations in other ways, is readily hydrolysed by trypsin preparations, whereas the protein prepared from disrupted virus by one or two precipitations with ammonium sulphate resembles "native" proteins in its resistance to proteolysis. It would appear that the products of disruption contain undenatured protein in an unstable state, they therefore resemble in many ways the "metaprotein" prepared by Theorell [1937] as a result of the removal of the lactoflavinphosphoric acid from Warburg's "yellow enzyme". In each case the protein which is made by splitting the starting material into a protein and a non-protein part is less stable than the intact molecule, but the unstable protein from tobacco mosaic virus can be kept at 0° for some weeks without apparent change. If however solutions which contain both protein and nucleic acid, but from which the SDS has been removed by dialysis, are kept, a gelatinous or slimy precipitate separates containing both protein and nucleic acid and only soluble if the pH of the fluid is raised above 8·5. The nucleic acid-free protein gives all the usual protein colour reactions and precipitates with the usual protein precipitants; its absorption spectrum resembles closely that of other proteins, i.e. there is an absorption maximum at 2750–2800 Å. and general absorption below 2500 Å. This supports the view that the intense absorption maximum at 2600 Å. which is found in tobacco mosaic and bushy stunt virus preparations [Bawden & Pirie, 1938, 2] is due to nucleic acid, for this absorption maximum is absent from the nucleic acid-free protein preparations.

Many attempts at fractionation have been made using acid, ammonium sulphate and similar agents but so far it has not been found to be heterogeneous if the incubation with SDS has been continued for a time sufficient to inactivate the virus completely. This observation sheds some light on the constitution of the protein of high molecular weight, which is the only recognizable component of a fully active virus preparation, for it seems to disintegrate into a nucleic acid and a protein that is too large to pass through a cellophane membrane but too small to sediment in a few hours in a centrifugal field of 17,000 times gravity. Bernal & Fankuchen [1937] have stated that tobacco mosaic virus preparations have not been made to give true crystals in vitro but that the submicroscopic rods which are responsible for the physical properties of virus preparations have an internal regularity which might be described as crystalline. It has been suggested [Bawden & Pirie, 1937] that these rods are artefacts made by the irreversible linear aggregation of the virus particles as they occur in the sap of young plants. Study of the disruption of tobacco mosaic virus preparations suggests that these units (which have as yet only been studied by filtration methods), from which the rod-shaped aggregates are built up, are themselves built from a number of

similar or identical proteins of normal molecular weight. The methods that have been used so far for studying these products are however too crude to give a definite result. Some attempts have been made to prepare antisera using the disrupted virus as an antigen but these have been unsuccessful.

SUMMARY

The kinetics of the disintegration of a tobacco mosaic virus preparation by sodium dodecyl sulphate are described and also the preparation and properties of the resulting unstable protein.

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CCXXIII. PYRUVATE OXIDATION IN BRAIN
IV. THE OXIDATION PRODUCTS OF PYRUVIC ACID

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DETAILED study of the biological oxidation of pyruvic acid is desirable owing to its important role as an intermediary metabolite in carbohydrate breakdown by tissue. A summary and discussion of some earlier work on pyruvic acid metabolism has been given in previous papers from this laboratory [Peters, 1936; 1937; McGowan, 1937].

Recently, several quantitative investigations have been made. Working on slices of rat brain, Weil-Malherbe [1937, 2] showed that under anaerobic conditions the amounts of lactic acid and CO_2 formed from pyruvic acid were quantitatively given by the Krebs dismutation [Krebs & Johnson, 1937, 1]:

$$2CH_3CO.COOH + H_2O \rightarrow CH_3CHOH.COOH + CH_3COOH + CO_2.$$
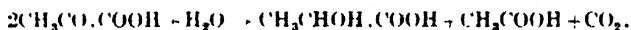
Only 80 % of the acetic acid was found, but the deficit could be accounted for approximately by formation of succinic acid. With brei, the tendency was to form more succinic acid at the expense of acetic acid.

In aerobic studies, McGowan [1937] determined the O_2 pyruvic acid ratio for pigeon brain brei, obtaining a value of 450 μl. mg., the theoretical for complete combustion being 635. This evidence of incomplete oxidation was confirmed by the high value found for the respiratory quotient, 1·3, as compared with the calculated value of 1·2 for complete oxidation. It was also shown that under these conditions the formation of a small amount of lactic acid accompanied the respiration, but not sufficient to account for the low value of the O_2 pyruvate ratio, assuming the lactic acid to arise from the Krebs dismutation.

The object of the experiments to be described was to continue the above work, which involves determining the amounts of lactic and acetic acids formed during the respiration, and finally to obtain a complete balance sheet for the oxidation of pyruvic acid under these conditions.

Experimental method

The procedure differs in one important respect from that of McGowan, namely that unwashed tissue has been used in all cases. The reason for this is that the pyruvate oxidase system in brain is to some extent destroyed by washing, with consequent fall in respiration, thus making difficult the accurate determination of the small quantities of lactic and acetic acids involved. This difference in technique leads to slight corrections in the increased O_2 uptake due to pyruvate. The appropriate corrections to be applied are dealt with separately in the sections dealing with the calculation of lactic and acetic acid formation.

The brain was prepared in the following way. Three pigeons were killed by decapitation and the brains dissected out. The membranes and cerebellum were removed and the rest thoroughly minced with a bone spatula on a warm (38°) plate. The brei was then transferred, in roughly equal quantities (350 mg.), to the experimental bottles.

Na pyruvate[1] was used as substrate. A pure sample of this was kindly supplied by Prof. R. A. Peters. A fresh solution in $M/10$ phosphate Ringer solution, pH 7·3, was made up for each experiment, such that 0·2 ml. contained about 10 mg. This amount was added to each bottle containing a total of 2·8 ml. fluid; final concentration $c.$ 0·03 M.

The respiration was measured as follows. The brain brei was introduced into the previously weighed bottles, each containing 1·5 ml. Ringer phosphate, pH 7·3, as used in previous experiments in this laboratory. After reweighing, the tissue was divided with a glass rod, 0·2 ml. of the Na pyruvate solution added and the total volume, including tissue, made up to 2·8 ml. with Ringer phosphate. In each experiment, four series of apparatus were used:

(1) Containing only tissue, stopped at the end of the equilibration period, i.e. just before the measurement of the respiration.

(2) Containing only tissue, not stopped until the end of the respiration.

(3) Containing tissue and pyruvate, stopped at the end of the equilibration period.

(4) Containing tissue and pyruvate, stopped at the end of the respiration.

Respiration was measured in O_2, using Barcroft-Dixon manometers. The usual technique of this laboratory was employed for the absorption of CO_2 (filter paper with $2N$ KOH). The rate of shaking was 108 oscillations per min. the bath being kept at $38°$.

Determination of the lactate/pyruvate ratio

The difference in lactate content of bottles 3 and 4 gives the amount of lactate formed from pyruvate during the respiration period. The difference in O_2 uptake of apparatus 2 and 4 gives the uncorrected O_2 uptake associated with the formation of this amount of lactate. At the beginning of the respiration period, the amount of residual lactate is about 0·6 mg. per g. tissue, and this falls to about 0·2 mg./g. at the end of the respiration (Table 1).

Table 1. *Residual lactic acid*

mg. per g. tissue

Exp.	Initial	Final	Difference
90	0·56	0·20	0·36
92	0·57	0·16	0·41
93	0·69	0·29	0·40
Average	0·61	0·22	0·39

This residual lactate disappearing is given by the difference between the lactate contents of bottles 1 and 2. The O_2 uptake associated with the oxidation of this 0·39 mg. lactic acid to pyruvic acid and beyond is 222μl. In bottles 3 and 4, the presence of pyruvate inhibits this lactate oxidation [Green & Brosteaux, 1936] with its corresponding O_2 uptake. Hence the observed increased O_2 uptake, due to disappearance of pyruvate, must be corrected by adding 222μl. per g. tissue. From this corrected O_2 uptake the amount of pyruvate disappearing during the respiration has been calculated, using the value for the ratio O_2 uptake/pyruvate disappearing $=450$ found by McGowan [1937]. In these experiments, the respiration was usually measured in duplicate or triplicate. The initial values quoted in Table I for residual lactate must not be confused with those observed by Kinnersley & Peters [1929; 1930] for lactate

[1] We are greatly indebted to Dr E. Stedman for recommending the use of Na pyruvate.

formed in brain soon after death. Amounts of the order of 1 mg. lactate per g. brain were formed from carbohydrate within about 1 min. after decapitation. Those in Table I are found roughly 90 min. after mashing, the smaller values being due presumably to oxidation of lactate to pyruvate in this system.

The estimation of lactate in the Barcroft bottles was carried out in the following way. Each bottle was treated with 1 ml. 25 % trichloroacetic acid to precipitate the proteins and prevent further respiration, and after ½ hr. the contents were filtered. The bottle was then washed out twice with 2 ml. 5 % trichloroacetic acid. To the combined filtrates were added 5 ml. 10 % $CuSO_4$ and 6 ml. of a 10 % $Ca(OH)_2$ suspension. After ½ hr. the volume was made up to 25 ml. and the bulky blue precipitate centrifuged off. 15 ml. of the centrifugate were used for each lactate estimation, according to the method of Friedemann & Kendall [1929], using the improved absorption tower of R. B. Fisher (private communication) and Na_2HPO_4 for aldehyde liberation [Lehnartz, 1928]. The average recovery of added lactate, using pure Zn lactate, was 98 %.

Table II summarizes the results of these experiments. In every case the value of the ratio lies between 4·9 and 5·4, the variations being within the experimental error of the method. The average value is 5·2 mol. lactic acid per 100 mol. pyruvic acid.

Table II. *Lactic acid formation*

Exp.	Duration in min.	Tissue mg.	O_2 uptake (μl.)		Pyruvic acid mg. (calc.)	Lactic acid mg.	L.A. P.A. molar %
			Uncorr	Corr.			
91	210	404	1901	1990	4·42	0·25	5·4
92	180	306	1557	1635	3·63	0·20	5·4
		343	1452	1531	3·40	0·19	5·4
		339	1444	1517	3·37	0·18	5·1
93	200	355	1581	1660	3·69	0·19	4·9
		362	1611	1691	3·76	0·20	5·1
94	195	283	1203	1267	2·82	0·15	5·3
		307	1305	1373	3·05	0·16	5·2

Determination of the acetate pyruvate ratio

As in the determination of lactate, the acetate production from respiration in pyruvate is equal to the difference in acetate content of bottles 3 and 4. There is a small amount of residual acetate formation, which is given by subtracting the acetate formed in 1 from that in 2. The net acetate production is therefore the difference between these two values. The small correction to be applied to the O_2 uptake is almost negligible, being only the O_2 absorbed in converting 0·39 mg. lactate into pyruvate, a value of about 50 μl. per g. tissue.

The estimation of acetate was based on the distillation method used by Weil-Malherbe [1937, 1, 2]. There are several substances which are volatile under the greatly reduced pressure employed. Free pyruvic acid, which is fairly volatile, is eliminated by conducting the distillation in the presence of phenylhydrazine [Weil-Malherbe, 1937, 2]: addition of pyruvic acid to the distilling flask did not increase the acidity of the distillate. Lactic acid is also slightly volatile under these conditions, but the amount distilled does not increase in proportion to its concentration [Virtanen & Pulkki, 1928]. In order to counteract any effect due to varying low concentrations, all distillations were carried out in the presence of a large excess of lactic acid, so that it was accounted for in the

blank estimation. The contents of the bulb of the Parnas apparatus were kept at about pH 2, at which reaction HCl is not liberated.

Each bottle was treated with 0·2 ml. 10% H_2SO_4 to precipitate protein and prevent further respiration. There was evidence that the use of sulphuric acid for this purpose did not affect the results, since similar values were obtained, in another connexion, when further respiration was prevented merely by placing the bottles in cold-store. When precipitation was complete—the bottles were usually allowed to stand overnight in the refrigerator—the contents were centrifuged, 2 ml. of the clear liquid being used for each estimation. The total amount of acetate was calculated from the volume of fluid in each bottle, tissue being counted as fluid volume, since the amount of solid matter is only about 15% of the wet wt. of the brain.

To the sample were added 10 ml. of a solution containing 1 ml. 10% phenylhydrazine hydrochloride, 1 ml. syrupy phosphoric acid and 5 g. NaH_2PO_4. 0·2 ml. (10 mg.) lactic acid was added. The mixture was allowed to stand for at least $\frac{1}{2}$ hr. before distillation, distilled water (2 ml.) being used to wash it completely into the bulb of the Parnas apparatus. Distillation in steam was carried out at 14–16 mm. pressure, an exact amount of distillate (55 ml.) being collected in each case. This was then titrated against freshly prepared $N/200$ CO_2-free NaOH, using bromothymol blue as indicator. The standard colour tint was a solution of sodium acetate to which a few drops of the indicator had been added. A clearly defined end-point at pH 7·6, sharper than with phenolphthalein, was obtained. Blank estimations were always performed, the usual value being about 0·25 ml. $N/200$ NaOH. Under these conditions, the recovery of added acetic acid, as shown in Table III, was 87·3%. Higher recoveries could be obtained by collecting more distillate, but this proved inconvenient.

Table III. *Recovery of added acetic acid*

Acetic acid mg.	$N/200$ NaOH (ml.)		Recovery %
	Calc.	Found	
0·650	2·166	1·898	87·6
		1·884	87·0
0·325	1·083	0·979	90·4
		0·972	89·8
0·163	0·542	0·469	86·6
		0·457	84·4
0·065	0·217	0·185	85·3

The procedure therefore differs from that of Weil-Malherbe in the following respects. Enzymic oxidation of pyruvic acid was arrested by the use of 10% H_2SO_4. A stock solution of NaH_2PO_4, phosphoric acid and phenylhydrazine hydrochloride—"acetate mixture"—was employed for all distillations, and proved of great convenience. All estimations were carried out in the presence of a large excess of lactic acid. Finally bromothymol blue was used as indicator.

Table IV. *Acetic acid formation*

Exp.	Duration in min.	Tissue mg.	O_2 uptake (μl.)		P.A. mg. calc.	Acetic acid mg.	H.Ac. P.A. molar %
			Uncorr.	Corr.			
80	170	327	1131	1147	2·55	0·42	24·4
81	175	392	1432	1452	3·23	0·55	25·1
82	240	383	1895	1914	4·25	0·71	24·5
		368	1891	1908	4·24	0·74	25·5
86	185	356	1329	·1346	2·99	0·53	25·9
		370	1367	1385	3·08	0·52	24·7
		377	1402	1421	3·16	0·51	23·8
87	185	348	1162	1180	2·62	0·44	24·6
		370	1217	1234	2·74	0·47	25·0

Acetic acid was identified by the method of Krüger & Tschirch [1929]. Four distillates were neutralized, evaporated to about 1 ml. and treated with 5 % lanthanum nitrate (0·5 ml.), $N/50\ I_2$ in KI (0·5 ml.) and 1 drop of 4N NH$_4$OH. On warming gently at first and finally boiling, the solution turned green and then blue, a characteristic blue precipitate separating out on cooling. This identified either acetate or propionate. Since the formation of the latter was unlikely, acetate was inferred.

Table IV summarizes the results of the experiments. It will be seen that the % ratio lies between 23 and 26 in every case, with an average of 24·8.

Balance sheet of pyruvic acid disappearance

Since the amount of lactic acid formed is so very small, the fate of pyruvic acid cannot be accounted for merely by the summation of the amounts which are completely oxidized or which follow the anaerobic Krebs dismutation. It cannot be argued that the observed formation of lactic acid is too small owing to reoxidation to pyruvic acid. As previously stated, the presence of pyruvic acid should inhibit completely the lactic dehydrogenase. Moreover, the Krebs dismutation cannot account for such a large production of acetic acid. To the above two processes

$$\text{I. } CH_3CO.COOH + \tfrac{1}{2}O_2 \rightarrow 2H_2O + 3CO_2.$$
$$\text{II. } CH_3CO.COOH + \tfrac{1}{4}H_2O \rightarrow \tfrac{1}{4}CH_3CHOH.COOH + \tfrac{1}{4}CH_3COOH + \tfrac{1}{4}CO_2.$$

must be added a third

$$\text{III. } CH_3CO.COOH + \tfrac{1}{2}O_2 \rightarrow CH_3COOH + CO_2.$$
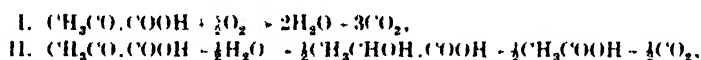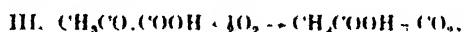
which will account for the excess of acetic acid formed over and above that produced by the Krebs dismutation.

The method of calculating the percentages of the above processes is as follows. 5·2 % lactic acid can only be derived from the Krebs dismutation; hence process II takes place to the extent of 10·4 %. Of the acetic acid formed, 5·2 % comes from the Krebs dismutation, so that the remaining 19·6 % is formed in process III. The amount of I can be deduced from a consideration of the O_2, pyruvate ratio.

450 μl. O_2 are required to oxidize 1 mg. pyruvic acid under these *in vitro* conditions. Process III oxidizes 1 mg. pyruvic acid with an accompanying 127 μl. O_2. Then, since this process takes place to the extent of only 19·6 %,

$$19\cdot6/100 \times 127 = 25\,\mu l.$$

of O_2 are absorbed. All the remaining O_2 is used up by process I. For complete oxidation, 1 mg. pyruvic acid requires 635 μl. O_2; it follows, then, that the amount undergoing complete oxidation is

$$425/635 \times 100 = 67\,\%.$$

The disappearance of pyruvic acid can thus be accounted for quantitatively by an appropriate summation of the three following equations. The extent to which each process takes place is represented by the accompanying percentages.

$CH_3CO.COOH + \tfrac{1}{2}O_2 \rightarrow 3CO_2 + 2H_2O$	67 %
$CH_3CO.COOH + \tfrac{1}{4}H_2O \rightarrow \tfrac{1}{4}CO_2 + \tfrac{1}{4}CH_3CHOH.COOH + \tfrac{1}{4}CH_3COOH$	10·4 %
$CH_3CO.COOH + \tfrac{1}{2}O_2 \rightarrow CO_2 + CH_3COOH$	19·6 %
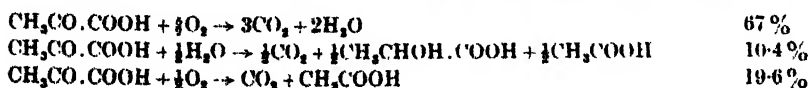
97 % of the pyruvic acid is accounted for.

From the above data, the respiratory quotient can be calculated:

$$\text{R.Q.} = \frac{5 \cdot 2 + 19 \cdot 6 + 3\,(67)}{\frac{1}{2}\,(19 \cdot 6) + \frac{1}{2}\,(67)} = \frac{225 \cdot 8}{177 \cdot 3} = 1 \cdot 28.$$

This agrees well with the experimental value of $1 \cdot 3$ found by McGowan [1937]; in fact if his one high value is excluded his average R.Q. is $1 \cdot 285$.

DISCUSSION

The preceding section provides us with a detailed knowledge of the ultimate fate of pyruvic acid when enzymically oxidized by pigeon brain. The importance of this knowledge is that we now have quantitative data which any theory of aerobic pyruvic acid oxidation must take into account. The results must, however, be interpreted with care.

The fact that a balance sheet can be drawn up by combining three equations in an appropriate manner does not necessarily mean that all three processes can take place independently of one another, or even that, as such, they take place at all. It may well be that the formation of lactic acid by the Krebs dismutation is independent of the oxidative processes. Nevertheless there is no direct evidence that lactic acid is formed in this manner in the system. It may possibly arise by some other process, so far unknown.

An "explosive" oxidation of pyruvic acid giving CO_2 and water, i.e. reaction I without any intermediary, seems most improbable. Several plausible theories of pyruvic acid oxidation have been proposed involving a series of oxidation products usually in the form of a cycle, as in the Toenniessen-Brinkmann [1930] scheme and the Krebs & Johnson [1937, 2] citric acid cycle. If there were no evidence to the contrary [McGowan & Peters, 1937], these might appear possible for aerobic oxidation in brain.

The possibility of acetic acid being an intermediate stage of pyruvic acid metabolism is very unlikely, since it has no noticeable effect on brain respiration, either in the presence or absence of pyruvate [McGowan & Peters, 1937; Elliott et al. 1937]. If the formation of acetic acid is not independent of other oxidative mechanisms, then it may conceivably arise from the breakdown of some initial stage of an unknown cycle. It is suggested that the following scheme might show the relationship between acetic acid formation and some general mechanism of pyruvate oxidation:

Pyruvic acid → (X) ⟋ Acetic acid
                       ⟍ Cycle regenerating some pyruvic acid, CO_2 and water being eliminated.
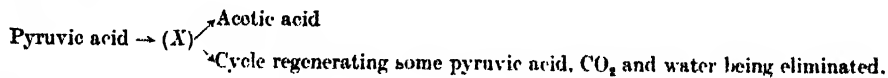
Hence a cyclical theory must involve some unknown stage (X).

It is clear from the balance sheet that if there is any formation of citric acid [Simola & Alapeuso, 1938], then it could only arise from about $3\,\%$ of the pyruvic acid apparently unaccounted for. Of course, the above remarks apply only to aerobic pyruvate oxidation in brain, and do not in any way embrace anaerobic oxidations such as are brought about by methylene blue.

SUMMARY

Lactic and acetic acids have been found in definite amounts when pigeon brain brei has respired in pyruvic acid. The quantities determined account for $30\,\%$ of the pyruvate disappearing, the remainder being oxidized completely to CO_2 and water, thus enabling a complete balance sheet to be drawn up.

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CCXXIV. THE EFFECT OF CERTAIN BACTERIAL TOXINS ·UPON SOME RESPIRATORY MECHANISMS OF ANIMAL TISSUES

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LITTLE work has hitherto been done to ascertain the effect of bacterial toxins upon physiologically active enzyme systems. Walthard [1934] reported that the addition of diphtheria toxin in bouillon to a rat liver preparation in a Barcroft micro-respirometer exerted no action upon the rate of O_2 absorption. On the other hand, Cross & Holmes [1937] reported that the power of isolated liver tissue of rabbits to form extra carbohydrate in the presence of various substrates is much lower for tissue obtained from animals suffering from diphtheritic toxaemia than for tissue obtained from normal rabbits. They also showed that in the presence of pyruvate the rate of O_2 uptake of liver slices obtained from a toxaemic rabbit is subnormal.

In this paper experiments are reported in which various toxins obtained from members of the *Clostridium* group of bacteria have been added to suspensions of actively respiring tissues in a Barcroft differential micro-respirometer.

EXPERIMENTAL

Corbet & Wooldridge [1936] pointed out the necessity for replication[1] and repetition of experiments made with the Barcroft differential manometer, especially when comparisons are made between results which do not differ widely. To record a result merely as the average of so many experiments without giving the standard error makes the acceptance of such results difficult. Our own experience with tissue respiration studies reinforces us in our view that results are often misleading unless experiments be both replicated and repeated and the final results expressed statistically.

Most of the work described has been done with suspensions in tap water of small intestine minced in a Latapie mincer after washing to remove intestinal contents. Other tissues have been used and the technique of their preparation is similar, the tissues being minced in the Latapie apparatus (liver, kidney, skeletal muscle) or ground in a mortar (brain, heart muscle), suspended in tap water and filtered through muslin in order to obtain a more homogeneous suspension. Owing to the fact that the minced tissues actively oxidized succinate, and to the advisability for replication of results, which was relatively easy with suspensions of finely minced tissue, the tissue slice technique was not followed in this work.

The tissues were obtained from guinea-pigs, and were used within 1 hr. of the death of the animal. The small intestine was employed to a great extent as

[1] By "replication" is meant the setting up of a series of identical experiments at one and the same time.

Cl. welchii is frequently the cause of intestinal trouble in certain animals. In the manometric experiments tissue was placed in each cup, and usually the only difference between the contents of the left and right cups was the addition of substrate to the latter. Many substrates have been tried but mainly succinate has been used. The results given are based on experiments lasting 40 min. at 37°, in which the rate of O_2 absorption was still proceeding linearly. There was usually a diminution in the rate after 1 hr. The experiments were continued for 2 hr. to make sure that their course was normal. The majority of our experiments were put up in quadruplicate or higher replication and each experiment was usually repeated at least 5 times. In the tables the results are expressed as means, with the standard errors given in brackets. In order to facilitate statistical analysis, the mean of the replicated results from any particular experiment was expressed as 100 for the O_2 absorption in 40 min. by a substrate alone in the presence of a particular tissue but in the absence of toxin or antiserum. (The actual results varied in the different experiments between $80 \mu l.$ and $150 \mu l.$ for succinate.) The figures for the O_2 uptake in the presence of toxin or antiserum were altered in the same proportion to facilitate comparison.

The micro-respirometers contained air and were shaken at 37°, the readings being started after 10 min. temperature equilibration. A roll of filter paper soaked in KOH solution, to absorb CO_2, was present in the centre cup of all manometric flasks.

The toxins and antitoxins used were obtained through the kindness and generosity of Dr O'Brien, Prof. Dalling and Dr Montgomerie of the Wellcome Physiological Research Laboratories, Langley Court, Beckenham, Kent, England. These toxins were prepared from broth filtrates of cultures of the appropriate anaerobe by precipitation with 70% ammonium sulphate, the deposit being dried *in vacuo*. The broth used was made from horse muscle with the addition of Parke Davis peptone and a large proportion of cooked meat in the case of *Cl. welchii*, types A and D, and of Witte peptone with only a small amount of cooked meat in the case of the type C organism. As a control for these toxins a similar preparation was made from the uninoculated broth used for *Cl. welchii*, type A, and this is alluded to in the tables and the text as the broth precipitate or broth precipitate control. The strength of toxin usually used in the mano- metric flasks was 0·4 mg. dried material per flask. The tissues used were usually washed and suspended in tap water but the contents of the flasks were main- tained at pH 7·4 by the addition of 0·5 ml. $M/20$ phosphate buffer. No change in pH occurred during the experiments. The average dry wt. of tissue (small intestine) added to the flasks was 14 mg., but it varied between 11 and 16 mg. and on occasion was as high as 20 mg. Where toxin and antiserum were used in the same cup they were mixed and allowed to stand for 15 min. before adding to the tissue.

Aerobic oxidations by minced small intestine. Krebs [1933; 1935] and others have reported no marked oxidative activity of small intestine with various substrates. Using aqueous suspensions of minced small intestine in the micro- respirometer we have been unable to show any definite oxidizing activity for any substrate other than succinate, *p*-phenylenediamine and dihydroxyphenylalanine (Table 1). Under our experimental conditions we were unable to show any activity with glucose, fructose, sucrose, lactate, pyruvate, fumarate, malate, oxalate, butyrate, oxaloacetate, citrate, formate, glycerol, alanine, serine, tryptophan, glutamate, aspartate, phenylalanine, glycylglycine, leucylglycine, glycyltryptophan or glycyltyrosine.

Table I. *Aerobic oxidations by small intestine*

Experimental details in the text. Each experiment with succinate and with *p*-phenylene-diamine was quadruplicated and with dihydroxyphenylalanine was triplicated. The *p*-phenylenedi-amine was used as the hydrochloride neutralized with NaOH. The mean values are given for the dry wt. of tissue used and for the "Q_{O_2}". The O_2 absorbed is due to the substrate in the presence of the tissue, the tissue being present in both cups, hence "Q_{O_2}" here represents the difference in true Q_{O_2} caused by substrate addition.

Substrate	No. of exps.	Dry wt. of tissue	Q_{O_2}
Succinate $M/120$	30	13·70	11·73 (s.e. 0·47)
p-Phenylenediamine $M/54$	12	15·85	10·33 (s.e. -0·20)
Dihydroxyphenylalanine $M/120$	5	16·78	3·65 (s.e. 0·40)

The effects of various bacterial toxins upon the aerobic oxidation of succinate in the presence of small intestine. Various toxins obtained from certain anaerobic bacteria were added to mixtures of succinate and small intestine in Barcroft micro-respirometers, and their effects upon the rate of aerobic oxidation observed. It will be seen from Table II that all the toxins inhibit the oxidation to some small

Table II. *The effects of various bacterial toxins upon the aerobic oxidation of succinate in presence of small intestine*

The figures given for the O_2 uptake represent the means, and their standard errors, obtained from the numbers of experiments mentioned. Details in text. The meaning of the symbols used is as follows. $S = M/120$ succinate, BP = control broth precipitate, A, C and D represent the toxins obtained respectively from *Cl. welchii*, types A, C and D. Oed., Sept., and Tet. represent the toxins obtained respectively from *Cl. oedematiens*, *Cl. septique* and *Cl. tetani*. All toxins were used in a concentration of 0·4 mg. dried product per manometric cup. s.e. = standard error, and P might be called the probability of significance. If $P^* < 0.01$ this is regarded as significant.

	S	S + BP	S + A	S + C	S · D	S · Oed.	S · Sept.	S + Tet.
No. of exps.	121	9	63	39	18	6	6	5
No. of manometers	467	27	237	141	69	23	24	18
Mean O_2 uptake	100·11	82·41	15·79	35·91	70·52	77·80	86·53	81·63
s.e.	0·46	1·04	0·35	0·71	1·56	1·69	2·35	2·15
Values for P compared with:								
(a) Succinate control	—	<0·01	<0·01	<0·01	<0·01	<0·01	<0·01	<0·01
(b) Broth ppt. control	—	—	<0·01	<0·01	<0·01	0·1–0·2	0·2	0·8–0·9

* For methods of statistical calculations see Fisher [1936].

extent but that a similar inhibition is induced by the "broth precipitate". Only the toxins of *Cl. welchii* produce inhibitions significantly greater than that of the broth precipitate, and of these inhibitions that induced by the type A organism is greatest and that by the type D least. The extent of the inhibition varies with the amount of toxin added, as is shown in Table III, but at the concentration chiefly employed in these particular studies, viz. 0·4 mg. toxin per cup, the inhibition by the toxins of the A, C and D types of organism are respectively 84·21, 64·09 and 29·48 % (Table II). The proportion of the α, β and ϵ toxins present in these three toxins is given in Table III and it will be seen that the α toxin is present in all. The β toxin is only present in the toxin from the type C organism and the ϵ toxin only in that from type D, and, in comparison with α toxin, their concentrations are high. These results suggest, therefore, that the inhibition of the succinate oxidation may be due to the α toxin, but that, in any case the inhibitory actions of the β and ϵ toxins must be small.

Table III. *The effect of the toxins of* Cl. welchii *in different concentrations upon the aerobic oxidation of succinate by small intestine*

The upper part of the table indicates the nature of the toxin preparations used, and the lower half gives the % inhibition produced by the various toxins in the concentration indicated in the first column. Experiments were made in quadruplicate and the standard errors were small.

Toxin	Type A	Type C	Type D	Broth ppt. control
Units toxin per 100 mg.				
α	12·5	Some	Some	0
β	0	100	0	0
ε	0	0	800	0
Inhibition of succinate oxidation (%)				
Mg. toxin per cup				
0·01	68	49	---	---
0·40	83	65	30	19
1·6	98	93	60	38
2·4	—	—	74	39

The effects of antisera upon the inhibition induced by the toxins of Cl. welchii. The oxidation of succinate by small intestine is inhibited by the presence of the toxins of Cl. welchii, but if various antisera are added as well as the toxin the inhibition induced by the toxin is considerably diminished by those antisera which contain antibodies for the α toxin. Table IV gives the results obtained

Table IV. *The effects of various antisera upon the inhibition by the toxin of* Cl. welchii. *type A, of the aerobic oxidation of succinate in the presence of small intestine*

The figures given in the table are the means, and standard errors, of five experiments each made in quadruplicate. The results have been adjusted so that the mean for the O_2 uptake due to succinate alone is 100. The α toxin present in the toxin used (0·4 mg. per cup) could be neutralized by a 1,10,000 concentration of antiserum or a 1 2800 concentration of the type D antiserum, as judged by mouse-inoculation experiments. S = succinate, AS = antiserum, N = normal horse serum, TX = toxin; other abbreviations as for Table II.

Antiserum	S	S + AS	S + TX	S + TX + AS	% inhibition		(TX + AS) − AS	% inactivation of TX
					TX	AS		
Welchii A, 1 10,000	100	88·70	15·00	47·60	85	11·3	41·10	51·7
S.E.	2·26	2·50	1·21	1·43				
Welchii C, 1 10,000	100·37	96·50	18·05	16·48	81·95	3·87	80·02	2·4
S.E.	2·23	2·28	1·71	1·35				
Welchii C, 1 4000	100·01	84·56	14·10	37·08	85·91	15·45	47·48	44·7
S.E.	1·37	1·61	0·80	1·41				
Welchii D, 1 10,000	100·65	92·95	17·85	16·20	82·80	7·70	76·75	7·3
S.E.	2·14	3·04	1·54	0·93				
Welchii D, 1 2800	100·25	85·22	13·55	49·92	86·70	15·03	35·30	59·3
S.E.	2·87	2·37	0·70	1·70				
Normal, 1 2800	100	91·90	11·20	8·40	88·80	8·10	83·50	6·0
S.E.	2·25	2·14	1·19	1·16				
Septique, 1 2800	100·25	88·55	13·65	9·40	86·60	11·70	79·15	8·6
S.E.	2·19	3·28	1·63	1·63				
Oedematiens, 1 2800	99·75	84·25	14·70	9·33	85·05	15·50	74·92	11·9
S.E.	1·84	2·44	1·09	1·10				
Tetani, 1 2800	100·27	85·10	13·85	8·35	86·42	15·15	76·75	11·2
S.E.	1·59	1·97	1·11	1·12				

with the toxin of *Cl. welchii* type A and various antisera, these sera being added in amounts equal to, or greater than, the concentration of the homologous antiserum which should neutralize the toxin, viz. 1/10,000. Of the antisera used only those for the three types of *Cl. welchii* contained antibodies for the α toxin. The antitoxic strengths of these three antisera were as follows: type A antiserum contained 300 units of α antitoxin per ml., type C antiserum contained 120 units of α antitoxin and 900 units of β antitoxin per ml. and type D antiserum contained 85 units of the α antitoxin and 275 units of the ε antitoxin per ml. From the results given in Table IV it is seen that when these three antitoxins are added in amounts equivalent in α antitoxin units they each reduce the inhibitory effect of the type A toxin by about half. The other antisera used and the normal serum produced no appreciable inactivation of the toxin. It will be noticed that all the sera themselves produced some small but significant inhibition of the succinate oxidation and this has been allowed for in calculating the extent of the

Table V. *The inactivation of the toxin of* Cl. welchii, *type A, by its homologous antiserum, as measured by the inhibition produced of the aerobic oxidation of succinate by small intestine*

Experimental details in the text. The figures given were obtained from replicated experiments and represent the O_2 absorbed.

Conc. of antitoxin	0	$\frac{1}{4} \times 10^{-4}$	$\frac{1}{2} \times 10^{-4}$	1×10^{-4}	2×10^{-4}	4×10^{-4}
No toxin	100	90	86	89	89	84
Toxin present	13	10	30	48	59	74

Table VI. *The effects of various antisera upon the inhibition by the toxins of* Cl. welchii, *type C and type D, of the aerobic oxidation of succinate in the presence of small intestine*

The figures given in the table are the means and standard errors of five experiments in the case of toxin C, and of fewer experiments in the case of toxin D, each made in quadruplicate. The results have been adjusted so that the mean for the O_2 uptake due to succinate alone is 100. The β toxin present in toxin C (0·4 mg. per cup) could be neutralized by a 1/3080 concentration of antiserum to the type C organism, while the ε toxin present in toxin D (1·6 mg. per cup) could be neutralized by a 1/66 concentration of antiserum to D (i.e. twice the actual concentration used), as judged by mouse inoculation experiments. S = succinate, AS = antiserum, Tx = toxin, N = normal horse serum and other abbreviations as for Table II.

| | | | | | % inhibition | | | % inacti- |
Toxin + AS	S	S + AS	S + Tx	S + Tx + AS	Tx	AS	(Tx + As) − AS	vation of toxin
Toxin C:								
Welchii A, 1/3080	100·20	81·95	30·92	48·35	69·28	18·25	33·60	51·5
S.E.	2·12	3·12	2·60	2·57				
Welchii C, 1/3080	100·11	85·46	37·99	51·26	62·12	14·65	34·20	43·95
S.E.	1·87	1·54	1·21	1·55				
Welchii D, 1/3080	100·32	82·58	30·42	39·01	69·89	17·73	43·58	37·7
S.E.	1·60	1·58	1·93	1·80				
Normal, 1/3080	99·82	86·93	29·42	25·20	70·40	12·89	61·73	12·3 ·
S.E.	2·12	2·20	1·92	0·96				
Septique, 1/3080	100·48	87·40	36·10	24·10	64·38	13·08	63·30	1·7
S.E.	2·22	2·91	1·09	1·43				
Toxin D:								
Welchii D, 1/134	100·33	90·2	58·66	70·5	41·67	10·13	19·70	52·7
S.E.	4·37	3·26	4·18	3·48				
Welchii A, 1/134	100·33	96·4	58·66	80	41·67	3·93	16·4	60·6
S.E.	4·37	4·31	4·18	3·29				

diminution of the inhibitory effect of the toxin on addition of serum. By varying the concentration of the antitoxin added the extent of the diminished inhibition is changed accordingly, as is shown in Table V. Results similar to those reported in Table IV for the toxin of *Cl. welchii*, type A, were obtained with the toxins of the organism, types C and D (Table VI). This table shows that the extent of the reduction of the inhibition due to these toxins is greatest with the antiserum for the type A organism, i.e. it is greatest with that antiserum containing the most α antitoxin. These results support the view that the inhibition of the succinate oxidation produced by the toxins of *Cl. welchii* is due to α toxin.

The effects of heated toxins upon the aerobic oxidation of succinate by small intestine. All the toxins used in this work are heat-labile, except that for the type D organism, the ε toxin in which is relatively heat-stable. If the inhibition of the succinate oxidation is due to any of these toxins except ε toxin, then it should be removed if the toxins are first heated to 80° for 15 min. The results given in Table VII indicate clearly that the small inhibitions induced by the

Table VII. *Comparison of the effects of toxins, before and after heating for 15 min. at 80°, upon the aerobic oxidation of succinate by small intestine*

The figures, which represent the amount of O_2 absorbed, are the means obtained from experiments replicated five or six times. The standard errors are given in brackets. The same intestinal preparation was used for a particular toxin, heated and unheated. Experimental details in the text.

Toxin	Succinate alone	Toxin added	Heated toxin added	Difference columns 3 and 4
Cl. welchii, type A	100 (3·39)	16 (2·68)	73·2 (3·06)	57·2
Cl. welchii, type C	100 (3·72)	34·6 (1·69)	66·6 (2·16)	32·0
Cl. welchii, type D	99·7 (0·87)	70·2 (2·2)	83·2 (3·01)	13·0
Cl. oedematiens	100·13 (4·85)	73·5 (4·87)	72·83 (4·73)	0·67
Cl. septique	100·13 (2·34)	80·17 (4·44)	78·33 (3·11)	1·84
Cl. tetani	100 (1·35)	94·17 (3·67)	95·60 (2·78)	1·43
Broth ppt. control	99·9 (1·54)	86·44 (2·58)	88·94 (3·20)	2·50

toxins from *Cl. septique*, *Cl. oedematiens* and *Cl. tetani* are unaffected by first heating the toxin but the inhibitions produced by the toxins from the three types of *Cl. welchii* are considerably lessened by pre-heating the toxin. This is further evidence suggesting that the inhibitory action is associated with the α toxin of *Cl. welchii*.

The effect of the addition of the toxin of Cl. welchii, *type A, upon other oxidations by small intestine.* The only substances which appear to be oxidized actively by aqueous suspensions of minced small intestine are succinate, *p*-phenylenediamine and dihydroxyphenylalanine (Table I). If the various toxins are added to mixtures of *p*-phenylenediamine and intestine in the micro-respirometer they all, including the broth precipitate control, inhibit the oxidation nearly 20 % but the toxins of *Cl. welchii*, type A, possesses a slightly more inhibitory action (*vide* Table VIII). The action of this toxin is significantly different from that of the broth precipitate control, but its magnitude is small in comparison with its inhibitory action on the succinate oxidation. Furthermore no inhibition significantly greater than that produced by the broth precipitate is obtained with the toxins of *Cl. welchii*, types C and D.

The addition of the toxin of *Cl. welchii*, type A, to dihydroxyphenylalanine ($M/120$) and minced small intestine diminishes the rate of oxidation to about 60 % of its normal value, an inhibition greater than that shown with *p*-phenylenediamine but only about half that registered with succinate (cf. Tables IX and II).

Table VIII. *Effects of bacterial toxins upon the aerobic oxidation of p-phenylene-diamine in the presence of minced small intestine*

Each experiment was made in quadruplicate. Abbreviations are the same as for Table II, but pp.d. = p-phenylenediamine ($M/54$).

Toxin	pp.d.	pp.d. +BP	pp.d. +A	pp.d. +C	pp.d. +D	pp.d. +Oed.	pp.d. +Sept.	pp.d. +Tet.
No. of exps.	10	6	5	5	5	5	5	5
O$_2$ uptake	100	82·78	72·58	86·37	81·3	79·23	82·15	82·66
s.e.	1·53	1·24	1·85	2·25	2·18	1·52	1·84	1·79
% inhibition	—	17·22	27·42	13·63	18·7	20·37	17·85	17·34
Values for P compared with:								
(a) Substrate control	—	<0·01	<0·01	<0·01	<0·01	<0·01	<0·01	<0·01
(b) Broth ppt. control	—	—	<0·01	0·6–0·7	0·4–0·5	0·5–0·6	0·7–0·8	>0·90

Table IX. *The effect of the toxin of* Cl. welchii, *type A, upon the rate of oxidation of* M/20 *dihydroxyphenylalanine by minced small intestine*

The experimental detail is the same as that described for succinate except that, owing to the slower rate of oxidation, the figures given represent the O$_2$ absorbed over a period of 80 min. Control experiments with "broth precipitate" gave small inhibitions comparable with that reported for succinate. s.e. = standard error.

	O$_2$ absorbed	s.e.	No. of exps.	No. of manometers
Substrate alone	100	2·12	5	16
Substrate + toxin	61·4	1·90	5	16

The effect of the toxin of Cl. welchii, *type A, upon the aerobic oxidation of succinate by various tissues.* Minced preparations of small intestine, skeletal muscle, heart muscle, liver, kidney and brain actively oxidize succinate in the Barcroft apparatus. The activity of heart muscle is very great, being 15 times greater than that of skeletal muscle. Despite their varying activities towards succinate the addition of the toxin of *Cl. welchii*, type A, to aqueous suspensions

Table X. *The effect of the addition of the toxin of* Cl. welchii, *type A, upon the aerobic oxidation of succinate by various tissues*

The results given are from single experiments replicated five times and are based on 40 min. readings, except for skeletal and heart muscle (20 min.). The experimental technique is described in the text. All inhibitions except that obtained in one of the brain experiments and that with brain and the broth precipitate control, are significant (i.e. P = <0·01). Three results are given with brain as they vary considerably from animal to animal. This variation does not occur with other tissues. The tissues were suspended in tap water. The standard errors are given in brackets.

			O$_2$ uptake		
Tissue	Dry wt. of tissue	Q_{O_2}	Succinate	Succinate + toxin or broth ppt.	% inhibition
(a) With toxin:					
Intestine	13·83	11·76	100·11 (0·46)	15·79 (0·35)	84·32
Skeletal muscle	38·00	8·37	100·5 (4·74)	16·5 (2·75)	84
Heart muscle	2·40	126·25	100·25 (3·33)	29·2 (3·54)	71·05
Liver	16·50	11·64	100·33 (3·56)	38·25 (4·77)	61·08
Kidney	7·80	35·00	99·8 (2·85)	25·8 (2·06)	74·00
Brain	7·80	7·20	100·25 (5·12)	109·75 (2·56)	− 9·5
Brain	17·30	13·20	99·83 (3·71)	78·17 (5·41)	21·66
Brain	12·50	12·78	99·60 (2·18)	52·00 (4·04)	47·60
(b) With broth ppt.:					
Intestine	14·47	12·06	100 (1·16)	82·41 (1·04)	17·59
Brain	8·0	13·80	99·9 (1·28)	95·75 (2·54)	4·15

of these tissues greatly inhibits the rate of oxidation of succinate with each tissue except brain. The inhibition is generally between 60 and 85 %, but with brain the results are indefinite although suggestive that some inhibition, less than that obtained with other tissues, occurs (Table X).

The effects of bacterial toxins upon a dried preparation of tissue enzymes. A dried preparation of "lactic dehydrogenase" was obtained from ox heart as described by Green & Brosteaux [1936]. This preparation was active to some extent with lactate and malate but it was much more active with succinate as its substrate. If the various bacterial toxins are added to mixtures of this enzyme preparation and succinate the rate of oxidation of the succinate is inhibited by the different toxins to an extent comparable with that shown with small intestine (cf. Tables II and XI). The toxins of Cl. welchii exert specific inhibitions greater than that

Table XI. The effect of bacterial toxins upon a dried preparation of succinic dehydrogenase obtained from ox heart

The abbreviations, experimental technique and method of presentation of results are similar to those described for Table II. A small amount of cytochrome c, prepared by the method of Keilin and Hartree [1937], was added to each cup. The number of experiments made was not large.

	S	S + BP	S + A	S + C	S + D	S + Oed.	S + Sept.	S + Tet.
No. of exps.	3	2	3	2	2	2	1	1
No. of manometers	10	6	10	6	6	6	3	3
O2 absorbed	100·22	82·00	19·64	36·16	57·50	83·00	87·33	75
S.E.	2·93	3·42	1·38	3·87	2·50	2·21	0·33	6·03
% inhibition	—	18·00	80·36	63·84	42·50	17·10	12·67	25·00
Values for P compared with:								
(a) Succinate control	—	<0·01	<0·01	<0·01	<0·01	<0·01	0·02–0·05	<0·01
(b) Broth ppt. control	—	—	<0·01	<0·01	<0·01	0·8–0·9	0·3–0·4	0·2–0·3

induced by the control broth precipitate. In one or two similar experiments carried out with this enzyme preparation but using lactate or malate as substrate no inhibition was produced by the presence of the toxin of Cl. welchii, type A. The rate of O2 absorption with these substrates was only half that given with succinate and as the experiments have only been done twice their preliminary character must be emphasized.

The effect of the toxin of Cl. welchii, type A, upon succinic dehydrogenase. The experiments described in this paper suggest that the α toxin of Cl. welchii inhibits the oxidation of succinate by its dehydrogenase. If the activity of the

Table XII. The effect of the toxin of Cl. welchii, type A, upon the anaerobic oxidation of succinate by its dehydrogenase

The dehydrogenase preparations used were minced small intestine and a dried tissue product prepared from ox heart after Green & Brosteaux [1936]. The usual anaerobic methylene blue technique was followed except that Thunberg tubes with hollow stoppers were employed and the succinate and methylene blue were tipped into the enzyme and toxin mixture after 10 min. anaerobic incubation at 45°. Reduction times (RT) in min. and the relative velocities (V) obtained from the reciprocals of reduction times are shown.

Exp.	Small intestine		Dried preparation	
	RT	V	RT	V
(a) Enzyme alone	∞	0	∞	0
(b) Enzyme + toxin	36·4	27	∞	0
(c) Enzyme + succinate	14·5	69	19	106
(d) Enzyme + succinate + toxin	10·4	96	24	83
(b + c) − d	—	0	—	23

enzyme in minced small intestine or in the dried tissue enzyme preparation be examined anaerobically in the presence of the toxin of *Cl. welchii*, type A, no marked inhibition is observed (Table XII). The addition of methylene blue, to act as an "oxygen carrier", to a mixture of small intestine, succinate and toxin in the Barcroft micro-respirometer should reduce the inhibition of the succinate oxidation by the toxin if the latter does not exert its effect upon the dehydrogenase. The results given in Table XIII show that in the presence of 1/3000 methylene blue the inhibition was reduced from 78 to 44 %.

Table XIII. *The inhibition of the aerobic oxidation of succinate by small intestine in the presence of the toxin of* Cl. welchii, *type A and methylene blue*

The O_2 absorbed in the presence of methylene blue (1/3000) was slightly more than without it, viz. 110 instead of 100. The experiment was made in quadruplicate. S = succinate, A = toxin of *Cl. welchii*, type A (0·4 mg.), MB = 1/3000 methylene blue and s.e. = standard error.

	S	S + A	S + MB	S + A + MB
O_2 absorbed	100	21·75	100	56
s.e.	1·58	0·48	1·58	2·97
% inhibition	—··	78·25	—	44
% reduction of inhibition	—	—	—	43·8

The effect of the toxin of Cl. welchii, *type A, upon the indophenol oxidase of small intestine.* If the presence of indophenol oxidase in small intestine is tested for with Nadi reagent in the way described by Keilin [1929], a very strong positive reaction is obtained. This reaction does not develop in the presence of cyanide but it is practically unaffected by the addition of the toxin of *Cl. welchii*, type A. Similar results were obtained with yeast and with heart muscle suspensions.

DISCUSSION

The results reported in this paper show that those bacterial toxins which contain the α toxin of *Cl. welchii* exert a specific inhibitory action upon the aerobic oxidation of succinate by aqueous suspensions of various minced tissues, e.g. small intestine, skeletal muscle, heart muscle, liver and kidney. A similar effect is experienced with a dried enzyme preparation made from ox heart by the method of Green & Brosteaux [1936]. A few preliminary experiments suggest that the aerobic oxidation of *p*-phenylenediamine by small intestine or of lactate or malate by the dried enzymic product is not inhibited by this toxin. Some inhibition, although only half as much as that with succinate, of the aerobic oxidation of dihydroxyphenylalanine by small intestine is experienced in the presence of the toxin. A number of other toxins of the *Clostridium* group of bacteria do not show any inhibition greater than that induced by the addition of a similarly prepared precipitate from uninoculated broth. The inhibitory action of the toxins appears to be specific to the α toxin of *Cl. welchii* for no toxin without the α toxin produces effects greater than the broth precipitate control, the inhibitory action is destroyed by heating to 80° for 15 min. and it is largely neutralized by the addition of antisera containing antibodies for the α toxin.

In comparison with its toxicity to the live animal the action of the toxin upon this enzyme system of tissues is low, although this may be due in part to our experimental conditions. Working on a basis of the presence of 70 % water in a live guinea-pig, the presence of 0·01 mg. toxin of *Cl. welchii*, type A, per g. dry tissue is lethal, whereas for the production of an inhibition of 80 % of the activity of the succinate system 1 g. dry tissue (small intestine) requires the

presence of 29 mg. of the toxin. Nevertheless, as an inhibitor of enzymic activity the toxin is potent.

If the inhibitory action of the toxin is examined under anaerobic conditions it is found that no such marked inhibition of the activity of succinic dehydrogenase is depicted. Neither can any inhibitory action of the toxin towards the activity of the indophenol oxidase of small intestine be demonstrated. These results, together with the aerobic oxidation of succinate by small intestine in the presence of toxin and methylene blue suggest that the inhibitory action of the toxin is not exerted directly either upon the dehydrogenase or upon the oxidase but may be associated with an interference to some intermediate link, possibly with an inactivation of some "carrier catalyst".

It has been clearly demonstrated that the α toxin of *Cl. welchii* interferes with a respiratory mechanism associated with the aerobic oxidation of succinate present in various animal tissues. Whether the pathogenicity of *Cl. welchii* is associated in any way with this inhibitory action upon an important respiratory mechanism of tissues is not known, but it is clear from recent progress in our knowledge of the physiological action of certain vitamins, e.g. lactoflavin or ascorbic acid, that the absence of factors associated with respiratory mechanisms of cells can lead to grave disease. Interference with enzymic systems necessary to the supply of energy to tissues might readily lead to subnormality of function, which may open the way to the development of a more active pathological condition. In this way a particular bacterial toxin might facilitate the subsequent invasion of the body by the toxigenic organism itself or by some other organism present at the time.

SUMMARY

1. The toxins obtained from *Cl. welchii*, types A, C and D, inhibit the aerobic oxidation of succinate by aqueous suspensions of minced small intestine.

2. This inhibition is reduced to a considerable extent by the presence of antisera which neutralize the α toxin of *Cl. welchii*.

3. The inhibitory action of the toxins is greatly reduced by heating for 15 min. at 80°.

4. The inhibitory action appears to be due to the α toxin.

5. The toxins of *Cl. oedematiens, Cl. septique* and *Cl. tetani* exert no action different from that induced by a precipitate prepared similarly from the broth medium.

6. This inhibitory action is not due to an effect upon either the succinic dehydrogenase itself or the indophenol oxidase, but may be due to an action upon some intermediate carrier catalyst at present unidentified.

7. It is suggested that the pathogenicity of *Cl. welchii* may be associated in part with this inhibitory action of its α toxin upon an important respiratory mechanism of tissues.

8. A brief discussion is given of the importance to invading bacteria of factors which will inhibit enzymic reactions of the host.

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CCXXV. SULPHYDRYL GROUPS AND ENZYMIC OXIDO-REDUCTION[1]

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In an attempt to correlate the observable physical changes that occur within the cell at mitosis with definite chemical processes, the author was led to formulate the working hypothesis [Rapkine, 1931] that cell division is accompanied by configurational changes of the cell proteins resulting in an increase of protein SH groups.

When it was later shown that iodoacetic acid reacts stoichiometrically with SH derivatives like cysteine and reduced glutathione [Dickens, 1933, 1, 2: Rapkine, 1933, 1] as well as with protein SH [Rapkine, 1933, 2] according to the equation

$$R - SH + ICH_2COOH = HI + R - S - CH_2COOH,$$
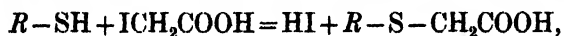
various attempts were made [Ellis, 1933; Runnström, 1935; Rapkine, 1937] to study the effect of this halogen acid on cell division.

In the meantime results published by various authors not only strengthened the belief that sulphydryl proteins (reduced—partially denatured(?)—proteins) may play in some manner an essential part during mitosis, but also lent more likelihood to the supposition that whenever the activity of a biological system proves to be inhibited by iodoacetic acid it is because this acid acts on SH groups contained in this system. Such are the results obtained on the influence of temperature, pH and concentration on the velocity of interaction of iodoacetic acid and the SH groups of cysteine, glutathione (GSH) and denatured proteins [Rapkine, 1933, 1, 2; 1936]. Also the work of Schubert [1936] has shown that the speed of interaction of iodoacetic acid with tertiary amines is a good deal lower than with SH derivatives. Finally it was possible to show that after having inhibited cell division in yeast cells, by carefully controlled action of iodoacetic acid, these cells could be made to divide again by adding to the culture medium SH compounds [Rapkine, 1937].

It was therefore thought desirable that as many protein systems as possible should be studied *in vitro* with respect to their sensitiveness to iodoacetic acid. Those which should prove to be appreciably inactivated by this acid were to be examined for SH and to see to what extent their activity depended on the integrity of thiol groups within their structure. As prototypes of such systems enzyme preparations appeared to be the most adequate.

The choice of particular enzymes must perforce be an empirical one, since very little is known about those which may be involved in the metabolism of the cell during its division. The choice, however, as to the class of enzymes need not be, as far as our own work on cell division is concerned, empirical. The results obtained in the first paper of this series on the sea-urchin egg [Rapkine, 1931] led us, indeed, to think that the period of predivision is characterized by dehydrogenation processes. Moreover, the lactic acid production in the sea-urchin egg

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[1] A preliminary note has already been published [Rapkine, 1938].

was correlated with the presence of SH groups. Our attention was therefore
directed to enzymes which catalyse dehydrogenation.

This paper is concerned with the enzyme which catalyses the oxido-reduction
between triosephosphate and pyruvate according to the following equation:

$$\text{Triosephosphate} + \text{pyruvate} \rightarrow \text{phosphoglycerate} + \text{lactate}.$$
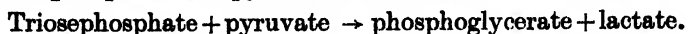
The presence of coenzyme I is necessary for this oxido-reduction to take place
[Meyerhof & Ohlmeyer, 1936; Euler *et al.* 1936]. A detailed study of the nature
of the enzyme was made by Green *et al.* [1937] who, in the course of their work,
have shown that it is one of a very small number of dehydrogenases that are
readily inhibited by fairly low concentrations of iodoacetic acid.

Although there exists already an extensive literature dealing with the thiol
groups of hydrolytic enzymes, and how the activity of these enzymes is influenced
by oxidation and reduction of their SH groups,[1] the only work of this kind on
dehydrogenases has recently been published by Hopkins & Morgan [1938] who
brought forward evidence showing that succinic dehydrogenase depends for its
activity on the presence of thiol groups.

In the work to be described in the present paper it will be shown that the
activity of the enzyme responsible for the oxidation of triosephosphate by
pyruvate depends on its state of oxidation-reduction. When oxidized by I_2 or
by oxidized glutathione (GSSG) its activity is greatly diminished. When re-
reduced by cysteine, reduced glutathione (GSH) or H_2S it is reactivated con-
siderably. Oxidized methylene blue is shown to have no effect whatsoever.
With the working hypothesis in mind that it is the SH of the enzyme proper that
is being oxidized and reduced, further experiments were made with Cu_2O which
is known to react particularly readily with thiol compounds [Hopkins, 1929;
Pirie, 1931]. In this case too the evidence obtained shows that the activity of the
enzyme can be nearly totally suppressed by Cu_2O and nearly fully regenerated by
treatment with H_2S.

However, in order to ascertain that in the experimental conditions to be
described the action of Cu_2O, as well as the action of oxidants and reductants, are
not concerned rather with the coenzyme than with the enzyme, experiments
were run in which the coenzyme was removed by adsorption before any subse-
quent treatment of the enzyme. The results obtained show that this is not the
case; the action of Cu_2O, oxidants and reductants concerns mainly (if not
entirely) certain active centres (SH) of the enzyme proper.

I. *Preparation of the enzyme and methods of estimating its activity*

The enzyme is essentially a dialysed aqueous extract of dried acetone powder
from rabbit skeletal muscle. It is conveniently prepared by the technique of
Meyerhof & Kiessling [1935] according to the directions of Green *et al.* [1937].
The method and the conditions of estimating enzymic activity were essentially
the same as those used by the latter authors. Since the oxido-reduction of triose-
phosphate and pyruvic acid results in the production of acid, the activity of the
enzyme which catalyses this reaction may be followed manometrically after
addition of $NaHCO_3$ to the mixture containing the enzyme, the substrate and
pyruvic acid. Since reduction of pyruvic acid to lactic acid involves no change
in acidic groups, the increase in CO_2 pressure is due to phosphoglyceric acid which
is formed on oxidation of triosephosphate. There is still however one more
condition to be observed if the CO_2 production is to serve as a means of measuring
the activity of the enzyme. Hexosediphosphate, which is used throughout these

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[1] Full references are to be found in an excellent critical review by Hellerman [1937].

experiments as the source of triosephosphate, is known to give rise to pyruvic acid via phosphoglyceric acid. Fluoride inhibits this reaction, and it must therefore be added in all experiments. Measurements were carried out at 25° and at 30° in the presence or absence of O_2.

II. *Experimental procedure*

(a) *Experiments on inactivation of the enzyme by oxidizing with GSSG.* Preliminary experiments have shown that fairly concentrated solutions of GSSG were necessary and the following procedure was adopted: 5 ml. enzyme solution were placed with 2·5 ml. $M/20$ GSSG (final concentration of GSSG is $M/60$) in a Thunberg tube. The tube was evacuated and kept in the water bath at 30° for 3–5 hr. A control experiment was run along identical lines, in which the GSSG was replaced by 2·5 ml. water. The tubes were then opened and 1 or 1·5 ml. of each mixture was pipetted into a Warburg manometer cup. The following reagents were then added: 0·1 ml. M pyruvate, 0·3 ml. $M/2$ NaHCO$_3$, 0·7 ml. $M/10$ NaF, and finally 0·4 ml. $M/10$ hexosediphosphate was placed in the side-tube. After temperature equilibration, the hexosediphosphate was tipped from the side-tube into the main compartment of the Warburg cup and the gas evolution was followed.

(b) *Experiments on inactivation by oxidizing with I_2.* The action of I_2 is very rapid, and there was no necessity for incubating the enzyme with I_2 for more than 30 min. As the enzyme solution contains reducing substances which react immediately with I_2, it is quite impossible to know what fraction of the I_2 added reacts with the enzyme itself. The procedure adopted consisted in adding I_2 to an aliquot part of the enzyme solution until its reduction became slow. The amount varied with the enzyme preparation, the final I_2 concentration varying according to the experiments from 0·003 to 0·009 N. The rest of the procedure was exactly the same as in (a) above.

(c) *Experiments on inactivation with I_2 and GSSG, and reactivation with H_2S, GSH and cysteine.* When I_2 was used as the oxidizing agent, the mixture of enzyme and I_2, after keeping in the water bath at 30° for not more than 30 min., was divided into two equal parts, one of which was reserved, whereas the other was treated with H_2S. The H_2S was then eliminated by a current of H_2. As usual a third equal portion treated similarly except for the addition of I_2 served as control. Still another control of fresh enzyme solution was run for the manometric measurements, in order to see the extent of change in activity (if any) the enzyme may have suffered.

When GSSG was the inactivator, the enzyme solution, after incubation with GSSG at 30°, was dialysed for 15 hr. against distilled H_2O at 0° through a cellophane membrane. The enzyme solution was now divided up into three equal lots, a, b and c. To a, $M/10$ GSH was added to make the final concentration $M/35$. To b an equivalent amount of $M/10$ cysteine and to c the necessary amount of water were added. The three lots were now placed in Thunberg tubes; these were evacuated and placed in the water bath at 30° for 3–4 hr. A portion of the mutase solution, which served as a control, was taken through all the stages of treatment, dialysis, incubation, dilution etc., but without added glutathione. Dialysis of the enzyme solution results in a marked loss of activity, and therefore for the manometric measurements another control experiment was run with fresh untreated enzyme solution (adequately diluted) which had been kept in the ice chest at 0°.

One other technical detail deserves mention. Since prolonged dialysis results in a marked loss of activity, a second dialysis after treatment with GSH or

cysteine was not practicable. It is therefore necessary, whenever manometric measurements are done in the presence of O_2, to take into account the absorption of gas due to the oxidation of the excess GSH and cysteine, which in the presence of the enzyme solution is not negligible.

(d) *Experiments on inactivation with Cu_2O and reactivation with H_2S.* To 10 ml. enzyme solution 200 mg. Cu_2O were added. The mixture was kept in the water bath at 30° for 15–20 min., with occasional stirring. It was then filtered and divided into two equal parts. One was reserved, while the other was treated with H_2S in the usual manner. As a control a third portion underwent the same treatment (incubation and H_2S) but without addition of Cu_2O. In most experiments however the control portion of enzyme solution was taken through the stage of incubation at 30° only, since experience has shown that treatment with H_2S does not in any way change the activity of the enzyme.

(e) *Experiments on inactivation and reactivation of the enzyme after treatment with charcoal to remove coenzyme I.* Merck "Medicinal" charcoal proved to be a good adsorbent of coenzyme I, provided that the charcoal was first washed with distilled water until the wash-water was no longer acid. The enzyme loses most of its activity after two charcoal adsorptions only. On addition of coenzyme I^1 the activity of the enzyme solution is fairly completely restored. Once the enzyme had been charcoal-treated, it was taken through all the stages of inactivation with GSSG, I_2 and Cu_2O. Reactivation experiments were only attempted on charcoal-treated enzyme which had been inactivated by I_2 and Cu_2O. Further experimental details will receive mention in the description of the particular experiments.

III. *Results obtained*

(a) *Oxidation and inactivation by GSSG.* Table I shows the results obtained in typical experiments when the enzyme is incubated with GSSG at various concentrations and for various periods of time at 30°.

Table I

After incubation the following amounts of the reagents were pipetted into the Warburg manometer cups for manometric measurements of the enzymic activity; 1·5 ml. treated or untreated enzyme, 0·4 ml. $M/10$ hexosediphosphate, 0·1 ml. M pyruvate, 0·3 ml. $M/2$ $NaHCO_3$, 0·7 ml. $M/10$ NaF. The hexosediphosphate was in the side-bulb of the cup and was only tipped into the main compartment after temperature equilibration. The amounts and concentration of the reagents added were, unless otherwise stated, always the same and need not afterwards be repeated.

Final conc. GSSG	Time of incubation hr.	Temp. of manometric measurements		μl. CO_2 in 30 min.
$M/100$	$1\frac{1}{2}$	25°	Enzyme + GSSG	171
			Control without GSSG	256
$M/100$	1	25°	Enzyme + GSSG	160
			Control	198
$M/60$	$5\frac{1}{2}$	25°	Enzyme + GSSG	11
			Control	157
			Fresh enzyme	146
$M/60$	$5\frac{1}{4}$	25°	Enzyme + GSSG	17
			Control	137
			Fresh enzyme	145
$M/60$	$4\frac{1}{2}$	30°	Enzyme + GSSG	45
			Control	102
			Fresh enzyme	111

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[1] I am indebted to Dr T. Cahn and Dr J. Houget for samples of coenzyme 1.

As is to be noted, fairly high concentrations of GSSG and rather long periods of incubation are necessary in order to oxidize and hence to inactivate the enzyme. The control which has been put through all the stages of treatment except for the added glutathione, suffers very little loss of activity as can be seen on comparing its activity with that of fresh mutase which had been standing in the ice chest at 0° until the manometric measurements were made.

(b) *Oxidation and inactivation by I_2.* I_2 reacts very quickly with the enzyme and the time factor is unimportant. As is seen in Table II it is the I_2 concentration which matters.

Table II

Vol. enzyme ml.	Final conc. I_2	Time of incubation at 30° (min.)	Temp. of manometric measurements		μl. CO_2 in 30 min.
1·2	0·0009 N	30	25°	Enzyme + I_2	137
				Control	256
1·3	0·003 N	10	25°	Enzyme + I_2	72
				Control	183
1·0	0·009 N	30	25°	Enzyme + I_2	40
				Control	198

(c) *The action of methylene blue on the enzyme.* It was desirable to know whether some other oxidant than GSSG and I_2 could be made to bring about an inactivation of the enzyme. Oxidized methylene blue was tried. Methylene blue (0·006 M) was added to the enzyme solution until there was no more reduction, even after the mixture was evacuated in Thunberg tubes. After 3 hr. incubation in the water bath at 30°, the necessary reagents were added and the activity of the methylene blue-treated enzyme was compared with a control (Table III). No inactivation whatsoever occurred.

Table III

Manometric measurements at 25°.

	μl. CO_2 in 10 min.
Enzyme + methylene blue	106
Control without methylene blue	110

(d) *Inactivation with GSSG and reactivation with GSH and cysteine.* As was mentioned in section II, reactivation of GSSG-treated enzyme entailed the elimination of excess GSSG by dialysis which in itself caused a loss of activity of the enzyme. The figures in Table IV illustrate this, as well as the superiority of cysteine over GSH in reactivating the enzyme. This has been met with throughout all the experiments.

Table IV

Incubated with GSSG for 5 hr. Dialysed for 15 hr. at 0°. Incubated with GSH and cysteine for 3½ hr. Manometric measurements at 25°.

	μl. CO_2 in 30 min.
Enzyme oxidized by $M/60$ GSSG, then dialysed	37
Oxidized, dialysed enzyme reduced by $M/35$ cysteine	159
Oxidized, dialysed enzyme reduced by $M/35$ G-SH	81
Control, dialysed	95
Fresh enzyme diluted suitably	127

(e) *Inactivation with I_2 and reactivation with H_2S.* As long as I_2 is made to act upon a muscle extract containing both enzyme and coenzyme, the inactivation is very marked, but the reactivation is rather feeble (Table V).

Table V

Incubated at 30° for 10 min. Final concentration of I_2 0·003 N. Measurements at 25°.

	μl. CO_2 in 20 min.
Enzyme + I_2	23
I_2-treated enzyme reduced by H_2S	45
Control	88

As will be shown below, the reactivation by H_2S may be nearly complete if the I_2 is made to act upon an enzyme solution from which coenzyme I has been removed by charcoal adsorption.

(f) *Inactivation by Cu_2O and reactivation by H_2S.* Although inactivation by Cu_2O is rapid and thorough, the time of contact and the amount of Cu_2O are, up to certain limits, not negligible (Table VI). The degree of reactivation of

Table VI

Enzyme in contact for 3 min. at 30° with Cu_2O before filtration of excess of the oxide. 50 mg. Cu_2O per 5 ml. enzyme solution. H_2S was passed for 5 min.

	μl. CO_2 in 30 min.
Enzyme + Cu_2O	85
Cu_2O treated enzyme decomposed by H_2S	160
Control (treated with H_2S)	257

Enzyme in contact for 20 min. at 30° with Cu_2O before filtration. 100 mg. Cu_2O per 5 ml. enzyme. H_2S passed for 15 min.

	μl. CO_2 in 30 min.
Enzyme + Cu_2O	74
Cu_2O-treated enzyme decomposed by H_2S	243
Control (not treated with H_2S)	268

Cu_2O-treated enzyme by H_2S also varies with time, and it was found that 15–20 min. were quite sufficient for optimum reactivation.

(g) *Inactivation and reactivation of enzyme after treatment with charcoal to remove coenzyme I.* The fact that coenzyme I is in some methods of its preparation precipitated as a Cu^+ salt, albeit in acid solution, served as a warning in the interpretation of the results obtained when Cu_2O is made to act upon muscle extracts containing both enzyme and coenzyme I. Is not the inactivation of an enzyme solution by Cu_2O due mainly to its combination with coenzyme I?

It was at once clear that the only way to answer this question, not only for Cu_2O, but also for the other inactivators, was to subject the enzyme to oxidation and reduction after removal of its coenzyme by charcoal adsorption. Preliminary experiments showed to what extent the enzyme suffers loss of activity on adsorption (Table VII). It is certainly not negligible, and the addition of coenzyme I in varying amounts restores, at best, about 80 % of the original activity. As for the residual CO_2 production by the complete system in presence of charcoal-treated enzyme without added coenzyme I, it cannot be due to dismutation of triosephosphate, since in the presence of pyruvic acid dismutation is completely

suppressed [Green *et al.* 1937]. It is very likely due to remaining traces of coenzyme I which have escaped adsorption, and which allow some oxido-reduction between triosephosphate and pyruvate to take place.

Table VII

Manometric measurements at 25°.

	mg. cozymase added		
	1·5	1·0	0·5
	μl. CO_2 in 30 min.		
	(1)	(2)	(3)
Charcoal-treated enzyme + hexosediphosphate + pyruvate + NaF	33	29	55
As above but with coenzyme I	126	130	146
Complete system with untreated enzyme	153	195	178

The results of Table VIII show clearly that under the experimental conditions described the action of Cu_2O cannot be exerted mainly on coenzyme I. If it were through some action on coenzyme I that inactivation of the enzyme by Cu_2O

Table VIII. *Action of Cu_2O on charcoal-treated enzyme*

Charcoal-treated enzyme solution was divided into 7 portions. To portions *a*, *b*, *c* and *d* Cu₂O was added (100 mg. per 5 ml.). After Cu₂O treatment (20 min. at 30°) the excess Cu₂O was filtered off, and whilst *a* and *b* were reserved, *c* and *d* were treated with H_2S. Coenzyme I was now added to *a* and *c*. To portion *e* was added a sample of coenzyme I which had been treated with Cu₂O, and the excess of the latter filtered off. To *f* ordinary coenzyme I was added, whilst no coenzyme was added to *g*. A normal enzyme solution (not charcoal-treated) was now divided into four other portions, *h*, *i*, *j* and *k*. *h* was the normal control; *i*, *j* and *k* were treated with Cu₂O (20 min. at 30°), and after filtration from excess Cu₂O *k* was reserved, whilst coenzyme I was added to *i*. To *j*, instead of coenzyme I, fresh enzyme solution (enzyme + coenzyme) was added. Of each of these various portions of enzyme 1 ml. was pipetted into Warburg manometer cups, and the necessary reagents were added for manometric measurements (see part (*a*) of this section).

		μl. CO_2 in 30 min.
(*a*)	Charcoal-treated enzyme + Cu₂O + coenzyme I	8
(*b*)	As above but without coenzyme I	19
(*c*)	Charcoal-treated enzyme + Cu₂O + H_2S + coenzyme I	102
(*d*)	As above without coenzyme I	51
(*e*)	Charcoal-treated enzyme + coenzyme I which has been treated with Cu₂O*	99
(*f*)	Charcoal-treated enzyme + coenzyme I	170
(*g*)	As above without coenzyme I	84
(*h*)	Normal (not charcoal-treated) enzyme	183
(*i*)	Normal enzyme + Cu₂O + coenzyme I	40
(*j*)	Normal enzyme + Cu₂O + fresh enzyme†	197
(*k*)	Normal enzyme + Cu₂O	45

* Coenzyme solution was in contact with Cu₂O (20 mg. per ml. for 20 min. at 30°).
† 0·5 ml. of fresh normal enzyme was added.

occurred, then we should expect the charcoal-treated enzyme to be reactivated, even after Cu₂O treatment, by mere addition of coenzyme I to the system. As is shown from Exps. *a* and *b*, this is not the case. Reactivation is only possible when the enzyme has been treated with H_2S (see Exp. *c* and *d*, Table VIII).

That Cu₂O does not react with coenzyme to any appreciable extent is also shown from *e*, where Cu₂O-treated coenzyme I was added to charcoal-treated

enzyme. Further evidence that Cu_2O acts mainly on some active centres of the enzyme proper is shown from Exp. h, i, j and k.

Action of I_2 on charcoal-treated enzyme. It was shown in part e of this section, that reactivation by H_2S of I_2-treated enzyme is rather small, so long as the I_2 is made to act upon ordinary enzyme solution. As will be seen from Table IX

Table IX

Final concentration of I_2 0·006 N. Manometric measurements at 25°.

	μl. CO_2 in 7 min.
Charcoal-treated enzyme + I_2 + coenzyme I	50
As above but without coenzyme 1	49
Charcoal-treated enzyme + I_2 + H_2S + coenzyme 1	113
Fresh mutase equally diluted	122
As above + I_2	29

good reactivation may be obtained if the I_2 is made to act upon an enzyme solution from which coenzyme I is removed. Moreover, it seems that the inactivation by I_2 of normal enzyme solution is more pronounced than in the case of charcoal-treated enzyme.

Action of GSSG on charcoal-treated enzyme. The results of Table X show the action of GSSG on charcoal-treated enzyme as compared with its action on normal enzyme (untreated with charcoal). The action of GSSG is much more

Table X

The enzyme was incubated with GSSG (final concentration $M/60$) for 4 hr.

	μl. CO_2 in 27 min.
Normal enzyme suitably diluted	228
As above + GSSG	181
Charcoal-treated enzyme + GSSG + coenzyme I	58
As above but without coenzyme I	18
Charcoal-treated enzyme + coenzyme I	147
As above but without coenzyme I	52

pronounced on charcoal-treated enzyme. It must be said that at present no explanation has been found to account for the difference of action of either I_2 or GSSG on normal and charcoal-treated enzyme preparations. Further work is being done in this direction.

DISCUSSION

The strict interpretation of the experiments described is that the enzyme responsible for the catalysis of the oxido-reduction between triosephosphate and pyruvic acid depends for its activity on its own oxidation-reduction state. Acted upon by oxidants, the activity of the enzyme is greatly diminished or nearly suppressed; once inactivated, the enzyme may recover its activity to a great extent by controlled treatment with reductants. In making the tentative hypothesis that these changes in activity of the enzyme are to be ascribed to the oxidation and reduction of the SH groups of the enzyme, one must necessarily cope with the long-standing objections which have already been formulated in the case of hydrolytic enzymes. To begin with, are the thiol groups involved at all? If they are, might there not be, side by side with these, other groups which are at least as essential for the activity of the enzyme? It is, of course, impossible at present to state definitely that there are no other active groups than the

thiol groups in the enzyme studied here. However, the hypothesis that thiol groups are mainly responsible for the activity of the enzyme can be supported by many arguments. Iodoacetic acid inhibits the enzyme readily and thoroughly. Although this acid has been shown to react with other groups, the speed with which it reacts with SH confers on this halogen acid, for practical purposes at any rate, the property of specificity towards thiol groups. The same can virtually be said of Cu_2O, in view of the conditions under which this acts upon the enzyme preparation. The only difference is that an enzyme completely inactivated by Cu_2O can be fully reactivated by H_2S, whilst inactivation by iodoacetic acid is irreversible.

In connexion with the reversible inactivation by GSSG, the arguments brought forward by Hopkins & Morgan [1938] in the case of succinic dehydrogenase are relevant. GSSG is a very mild oxidizing agent, and the fact that the $GSSG \rightleftharpoons 2GSH$ system is very negative in the potential scale makes it very likely that, as an oxidant for proteins, GSSG is specific for the thiol groups of the protein. There is further evidence for this. Hopkins [1925] has shown that in the oxidation of the reduced thermostable residue of tissue by GSSG, the resulting GSH formed is a measure of the pre-existing SH of the proteins. This was later confirmed on reduced heat-coagulated egg-white [Rapkine 1933], where it was, moreover, shown that the amount of pre-existing fixed SH of the protein as measured by the iodoacetic acid technique is identical with that obtained by Hopkins's method. Finally, Mirsky and Anson [1935] proved conclusively that the total number of fixed SH groups in a protein can be approximately measured by the total amount of hydrogen transferred to the oxidant (cystine).

The action of oxidized methylene blue on the enzyme studied here is again in harmony with the thiol group hypothesis. Methylene blue is, like GSSG, a mild oxidizing agent, and although it is high up in the scale of redox potentials it is known to react extremely slowly with thiol groups. This accounts for the ineffectiveness of methylene blue in inactivating the enzyme.

The action of I_2 needs hardly any comment. I_2 is by no means a mild oxidizing agent. Although it oxidizes SH to SS it cannot be said that it reacts readily only with SH groups. The action of I_2 on the enzyme must therefore be considered only in conjunction with the action of other substances such as GSSG, methylene blue and Cu_2O. However, it must be pointed out that inactivation of the enzyme by I_2 is to a large extent reversible by H_2S, especially if the I_2 is made to act on a coenzyme-free (charcoal-treated) preparation of the enzyme.

The behaviour of GSSG towards charcoal-treated enzyme is rather curious. Unless other substances present in the normal enzyme preparations interfere in some manner with the action of GSSG, it is perhaps not unreasonable to think that the coenzyme exerts in some way some protective influence on the enzyme. Once the coenzyme is removed by charcoal, GSSG inactivates the enzyme much more quickly. Whatever the explanation may be, account should be taken of these results whenever similar experiments of oxidation and reduction are carried out on enzymes which require, for their activity, the presence of a coenzyme.

This paper deals with the reversible inhibition of an enzyme which takes part in the first stages of glycolysis. It is therefore not out of place to compare the results obtained on this particular enzyme with those obtained previously by other authors on the effect of inhibitors on glycolysis by whole muscle extracts. Thus Lipmann [1934] showed that Cu in small amounts (10^{-5} to $10^{-4}M$) activates muscle glycolysis, whilst in concentrations of 10^{-4} to $10^{-3}M$ it inhibits

glycolysis. Previously the same author [Lipmann, 1933] had shown that the inhibition of glycolysis by dichlorophenol-indophenol and O_2 could be removed by ascorbic acid and N_2.

Wagner-Jauregg & Rzeppa [1936, 1, 2] obtained evidence that the copper inhibition described by Lipmann [1934] may be reversed by GSH, cysteine, coenzyme I and coenzyme II. More recently a detailed systematic study on the reversible inhibition of muscle glycolysis was made by Gemmill & Hellerman [1937]. Besides metallic compounds the authors also used I_2 as an inactivator of glycolysis, the reactivants used being cysteine, GSH and ascorbic acid. In this same paper [Gemmill & Hellerman, 1937] mention is made of unpublished experiments by Leövey (carried out in 1936) in which the author shows that the conversion of pyruvate into lactate in rabbit muscle pulp is inhibited by I_2 and quinone and restored by H_2S.

The results just enumerated make it tempting indeed to suggest that precisely the enzymic system dealt with in the present paper is one of the systems with which the above-mentioned researches on glycolysis was concerned.

SUMMARY

1. The enzyme which catalyses the oxido-reduction between triosephosphate and pyruvate is inactivated by oxidation with GSSG. If now the excess GSSG is dialysed away, the enzyme can be reactivated by GSH or cysteine, the latter being more rapid in its action.

2. The enzyme is inactivated by I_2. Such an inactivated enzyme can, to a large extent, be reactivated by H_2S. The degree of reactivation is greater if the I_2 is made to act upon an enzyme preparation from which coenzyme has been removed by charcoal adsorption.

3. Methylene blue exerts no influence on the activity of the enzyme.

4. Cuprous oxide completely inactivates the enzyme. Its activity can, however, be fully recovered on regenerating the enzyme by H_2S.

5. It is shown, using charcoal-treated enzyme preparations, that the action of Cu_2O, oxidants and reductants is mainly concerned with active groups of the enzyme itself.

6. The changes in activity of the enzyme under the influence of Cu_2O, oxidants and reductants are ascribed mainly to SH groups. The oxidation or combination of these groups results in an inactivation of the enzyme. The re-reduction or regeneration results in a reactivation of the enzyme. This hypothesis is discussed.

7. The work just reported is discussed in the light of work done by other authors on glycolysing extracts.

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CCXXVI. THE METABOLISM OF ADENINE COMPOUNDS BY *BACT. COLI*

BY MARJORY STEPHENSON AND ARTHUR REGINALD TRIM

WITH A MICRO-METHOD FOR THE ESTIMATION OF RIBOSE

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THE importance of nucleic acid derivatives in cell metabolism is becoming increasingly apparent; one may instance the function of muscle adenylic acid in fermentation and its presence as a constituent of coenzymes I and II; the pyrimidine ring occurs in cocarboxylase and uracil has been shown by Richardson [1936] to be essential for the anaerobic growth of *Staph. aureus*. In the metabolism of bacteria it has recently been shown that muscle adenylic acid prevents the loss of activity of *dl*-serine deaminase from a strain of *Bact. coli* [Gale & Stephenson, 1938], whilst adenosine behaves as a coenzyme for aspartase II prepared from the same organism [Gale, 1938].

As a preliminary to studying the mode of action of adenine compounds as coenzymes in the metabolism of bacteria the present study was undertaken in order to elucidate the changes which they themselves undergo in the presence of the enzymes of the organism. Previous studies on various bacteria (including *Bact. coli*) have shown that adenine and guanine are present as nucleotides, nucleosides and free bases in the acid-soluble extracts of the cells and that adenine compounds are more than ten times in excess of guanine compounds. On autolysis of the cells nucleic acid nitrogen decreases and nucleotides, nucleosides and free bases increase [Mesrobeanu, 1936].

It has also been shown that in the case of *Bact. coli* there exist within the bacterial cell enzymes decomposing adenine compounds and that muscle adenylic acid is dephosphorylated and deaminated by various organisms of the Bacteriaceae. Hypoxanthine was identified as the end product of the action of *Bact. coli* on adenine, adenosine, inosine and inosinic acid, and adenyl pyrophosphate was shown to be a cell constituent of the same organism [Lutwak-Mann, 1936]. It now seems probable that the acid-soluble purine derivatives found in the cell originate from the nucleic acid. It has been shown that yeast decomposes its own nucleic acid autolytically and that the adenylic acid thus formed (adenosine-3-phosphoric acid) is rapidly dephosphorylated giving adenosine; the latter can be phosphorylated in its turn by the enzymes of the same organism giving adenosine-5-phosphoric acid ("muscle adenylic acid") and also the di- and tri-phosphates functioning in fermentation [Ostern *et al.* 1937; 1938].

In the present paper the same strains of *Bact. coli* as those employed in previous work were used; three types of decomposition were studied: (1) the dephosphorylation of muscle adenylic acid; (2) the deamination of adenine, adenosine and adenylic acid; (3) the removal of ribose from adenosine and adenylic acid.

<div align="center">METHODS</div>

The organisms were grown in flasks of tryptic caseinogen digest broth for 12–14 hr. and centrifuged and washed with water in the usual way. The strength of the bacterial suspension was estimated by the photoelectric turbidimeter [Clifton *et al.* 1935]. The inorganic phosphate was estimated by the method of Fiske & Subbarow [1925], ammonia by the method of Parnas, and ribose by the method described in the appendix. The rates of dephosphorylation, deamination and liberation of ribose are generally expressed as μl. ammonia, phosphate or ribose per mg. dry wt. bacteria per hr. $\left(\mu l. = \frac{\text{mol. wt. in mg.}}{22,400}\right)$. Deamination is also expressed in Q_N (μg. nitrogen/mg. bacteria/hr.). The following stock solutions were used. Phosphate buffer pH 7·7 $3M$/450. Adenine 0·602 mg./ml. ($3 \times 33\,\mu$l./ml.). Adenosine 1·15 mg./ml. ($3 \times 33\,\mu$l./ml.). Muscle adenylic acid (adenosine-5-phosphoric acid) neutralized with NaOH 1·54 mg. ml. ($3 \times 33\,\mu$l. ml.). *d*-Ribose 0·670 mg./ml. ($3 \times 33\,\mu$l./ml.). Bacterial suspensions 3–15 mg. dry wt./ml. as convenient.

<div align="center">*The decomposition of adenylic acid*</div>

Adenylic acid is dephosphorylated and deaminated almost simultaneously, but in all cases so far tried the former process appears to precede the latter.

Exp. 1. 5 ml. each of stock phosphate, adenylic acid (2 × stock) and bacterial suspension (3 mg./ml.) were incubated at 37°; 2 ml. and 1 ml. for the estimation of
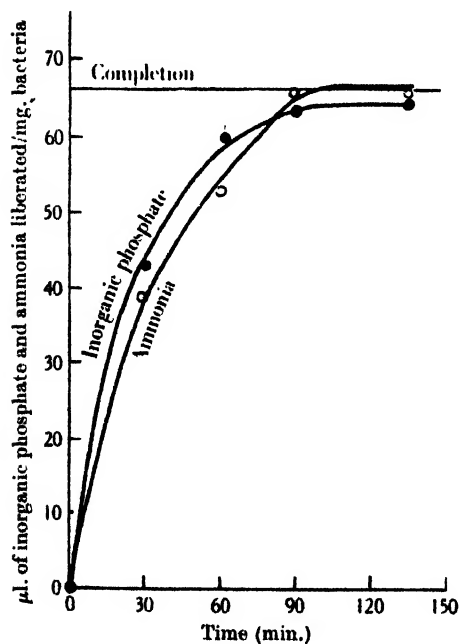
Fig. 1. The dephosphorylation and deamination of muscle adenylic acid by *Bact. coli* I.
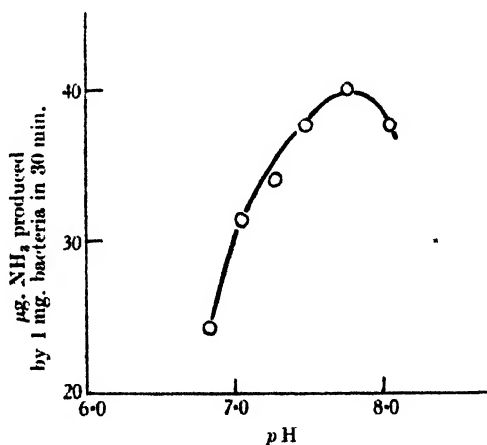
Fig. 2. Effect of pH on the deamination of adenosine by *Bact. coli* I.

ammonia and phosphate respectively were withdrawn at intervals; the results are shown in Fig. 1. The rate of deamination in this experiment corresponds to

$Q_N = 24$. The rate of these reactions varies only slightly with pH increasing by 12 % between pH 6·5 and 8·0 after which it falls off. From the pH curve for the deamination of adenosine (Fig. 2) it appears that the deamination of adenylic acid is controlled by the dephosphorylation and what is actually being measured is the effect of pH on dephosphorylation.

The action of inhibitors

The action of various inhibitors was tested in the hope of separating the dephosphorylation from the deamination. The results are given in Table I.

Table I

	% inhibition	
Inhibitor	Dephosphory-lation	Deamination
1. Phenol 1%	0	100
2. KCN M/500	100	100
3. KCN M/1000	100	100
4. NaF M/50	60	66
5. Iodoacetate M/300	0	0
6. Toluene (saturated sol.)	33	90
7. Chloroform (saturated sol.)	100	100

In the cases of 1, 6 and 7 the bacteria were exposed to the poison for 30 min. at room temperature and then centrifuged and washed before use: 2, 3, 4 and 5 were present during the reaction.

The only useful inhibitor found was 1 % phenol which effects a clean separation of the two mechanisms; the phenol-treated and control organisms are washed twice with distilled water before use.

Exp. 2. 4 ml. each of adenylic acid phosphate and phenol-treated bacteria (6 mg./ml.) were incubated for 30 min. at 37°; 2 ml. were then withdrawn for P and 1 ml. for NH_3 estimations. The remainder was then divided into two 4 ml. samples A and B. To A was added 0·26 ml. fresh bacterial suspension 15 mg./ml. so that the preparation contained 1 mg./ml. fresh bacteria; to B 0·26 ml. water was added. After 1 hr. further incubation the phosphate and ammonia estimations were repeated; the usual blank estimations were done (see Table II). Phenol treatment has no effect on dephosphorylation; when rates were measured with phenol-treated and untreated bacteria superimposable curves were obtained.

Table II

Time in min.		NH_3–N μl./2 ml.	Phosphate P μl./2 ml.
30	(Phenol-treated organism)	<8	47·6
90	(Phenol-treated organism)'	<8	47·2
90	(Phenol-treated + fresh organism)	46·4	47·2

Complete deamination and dephosphorylation corresponds to 66 μl./2 ml.

The decomposition of adenosine

The adenosine used was obtained from Georg Henning (Berlin) and gave the following figures on analysis:

C 45·11 %, H 4·90 %, N 25·4 % (calculated C 45·10 %, H 4·86 %, N 26·2 %).

Deamination. Adenosine is deaminated more rapidly than adenylic acid, especially by strain I, the difference being less marked in strain II (see Table III).

Table III

	Strain I	Strain II
Average Q_N adenylic acid	20	13
„ adenosine	104	24

Phenol treatment completely inhibits deamination by both strains.

The effect of pH is much more marked in the case of adenosine than in that of adenylic acid (see Fig. 2), the optimum being at pH 7·75. These facts are consistent with the view that adenosine is the compound actually deaminated.

Removal of ribose

The method used for the estimation of free ribose is described in the appendix. Having there shown that $10 \mu g.$ ($1·5 \mu l.$) ribose can be accurately estimated after the removal of the bacteria it became possible to study the liberation of this pentose from adenosine.

Exp. 3. 1 ml. each of stock adenosine phosphate buffer and bacterial suspension (3 mg./ml.); 1 ml. of this mixture contains 33 $\mu l.$ ($221 \mu g.$) ribose in the form of adenosine. Control exp. in which adenosine is replaced by water. Incubation at 37°.

1 ml. was withdrawn and deproteinized with 3 ml. $0·2 N$ HCl and 2 ml. of the filtrate used for the estimation of ribose, this is equivalent to $111 \mu g.$ ribose which requires 2·00 ml. ceric sulphate (Table IV).

Table IV

	Time in min.	Ceric sulphate ml.
Control	0	0·36
Exp. A	0	0·54
„	60	0·50
Exp. B	0	0·52
„	60	0·55

From the results in Table IV it seems that either no ribose is liberated in the experimental period or it is immediately decomposed to substances which do not reduce ferricyanide; Exps. 4 and 5 show that the latter alternative is correct.

Exp. 4. Six test tubes each containing 0·5 ml. stock adenosine, 0·5 ml. water and 3·0 ml. $0·2 N$ HCl were heated in a boiling water bath; at various intervals a tube was removed in which the total free ribose was estimated in the usual way; the results are given in Fig. 3. From this it is seen that 90 % of the theoretical amount of ribose is liberated from adenosine and can be estimated after 2 hr. acid hydrolysis. In order to raise the yield of ribose, stronger acid and longer hydrolysis were employed without effect. From the analytical figures for the adenosine used it seems unlikely that this deficit is due to impurity,

Table V

Time in min.	Ribose added $\mu g.$	Ribose estimated $\mu g.$	Furfuraldehyde test
0	100	99	0
30	100	93	0
60	100	97	(-)
120	100	104	(+)
180	100	98	(+)

though a control in which 100 μg. ribose were heated under the same conditions shows a higher recovery of ribose and a slight furfuraldehyde reaction by the aniline acetate test (Table V).
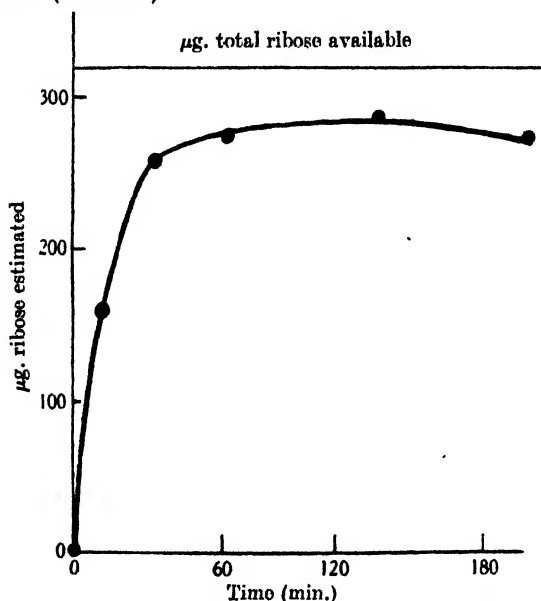
Fig. 3. Hydrolysis of adenosine by 0·2 N HCl at 100°.

We next tried to determine whether the ribose is actually split off from the adenosine as a result of the bacterial action.

Exp. 5. Equal vols. of stock adenosine, phosphate buffer (3M/450 at pH 7·7) and bacterial suspension 3 mg./ml. were incubated at 37° (1) aerobically, (2) anaerobically; 2 ml. samples were removed at intervals and 6 ml. 0·2 N HCl added, shaken with kieselguhr and filtered; the ribose was estimated in the filtrate after 2 hr. acid hydrolysis (A); 2 ml. of the filtrate without acid hydrolysis were used as control (B). $A - B$ ($=C$) is the ribose present as adenosine.

If C_0, C_{15} and C_{30} etc. are the quantities of ribose found after the action of the bacteria for 0, 15 and 30 min. C_0–C_{15} is the amount of ribose split off from adenosine in 15 min. as the result of bacterial action.

Table VI

Time min.	μg. ribose removed/mg. bacteria	
	Aerobically	Anaerobically
0	0	0
15	42	45
30	76	76
60	182	177

Total available ribose = 182 μg. (90 % of theoretical, see Exp. 4).

Table VI shows that in 60 min. 100% of the ribose of adenosine has disappeared yet no ribose can be detected in the culture fluid at any period as shown in Exp. 3. This implies that ribose must be destroyed as quickly as it is liberated, so a comparison was made of the rate of destruction of free ribose and that of ribose combined in adenosine.

Exp. 6. 2 ml. stock buffer and 2 ml. bacterial suspension (3 mg./ml.) were incubated (*a*) with 2 ml. stock adenosine, (*b*) 2 ml. stock ribose.

2 ml. samples from (*a*) and (*b*) were removed at intervals and the ribose in each estimated in (*a*) after acid hydrolysis (as in Exp. 5) in (*b*) direct. From these results the rate of decomposition of ribose (*a*) as adenosine, (*b*) as free ribose were determined both for organisms grown on broth and on glucose broth. The results appear in Table VII.

Table VII

Rate of fermentation of ribose µg./mg. bacteria/hr.	Free	As adenosine
Organism grown on plain broth	15	198
,, ,, glucose ,,	20	204
Both inhibited 100% by treatment of organism with 1% phenol.		

The rate of disappearance of ribose in combination as adenosine being so much greater than when free, confirmation of the phenomenon was sought by the manometric measurement of the fermentation. The course of the fermentation was followed by measuring the total gas evolved in bicarbonate buffer, which may be CO_2 or H_2, and also gas liberated by fermentation acid. The experiments were done in Warburg manometers.

Exp. 7. Each manometer contained 3 ml. total liquid.

0·2 ml. *M*/50 substrate (side bulb),
1 ml. bicarbonate buffer *M*/13,
1 ml. bacterial suspension (3 mg./ml.),
0·8 ml. water.
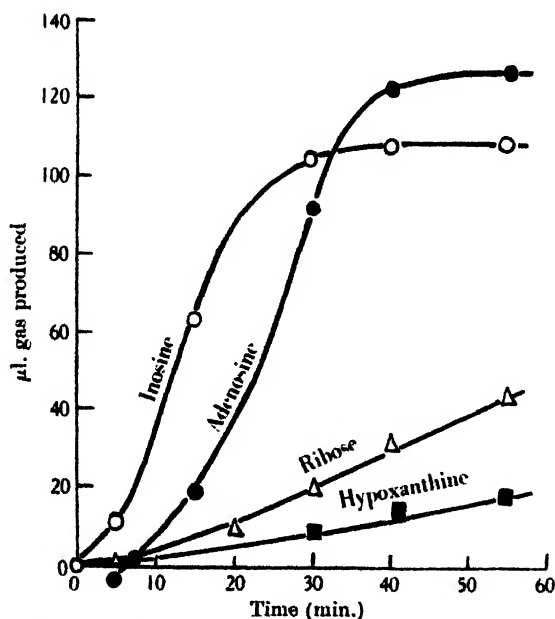
Fig. 4. Total gas produced in bicarbonate buffer from adenosine, inosine and free ribose (*Bact. coli* 1)

The usual controls were performed. Gas production from the following substrates was measured: adenosine, inosine, *d*-ribose and hypoxanthine; the results are given in Fig. 4. From this it is seen that the rate of fermentation of free

ribose is about 0·1 that of ribose combined in adenosine and in inosine. (The rate of fermentation of adenosine is the same in the presence and absence of phosphate.) The results of Exp. 6 are therefore corroborated and the disappearances of ribose in the free state and in adenosine are seen to be due to fermentation.

The deamination of adenine

Adenine is very slowly deaminated (Q_N 3–5); this rate is increased two- to six-fold by the presence of adenosine or inosine.

Exp. 8. Solutions were made up as in Table VIII; 2 ml. from each solution were removed at intervals and the NH_3-N estimated; the results are given in Table IX.

Table VIII

	ml.	ml.	ml.	ml.	ml.
Bacterial susp. 3 mg./ml.	3	3	2	3	2
Adenine stock	3	3	0	3	0
Adenosine stock	0	0·3	0·2	0·6	0·4
Water	1·5	1·2	2·8	0·9	2·6
Buffer 3 M/225	1·5	1·5	1·0	1·5	1·0
Total vol. ml.	9	9	6	9	6
No. of exp.	1	2	2a	3	3a

Table IX. NH_3-N

Exp. no. ...	1		2		2a		2–2a		3		3a		3–3a	
Time min.	mg.	μl.	mg.	μl.	mg.	μl.	mg.	μl.	mg.	μl.	mg.	μl.	mg.	μl.
0	<0·005	<8	<0·005	<8	<0·005	<8	0	0	0·005	8	<0·005	<8	0	0
30	<0·005	<8	0·0279	44·5	0·005	8	0·0274	36·5	0·035	56	0·010	16	0·025	40
90	0·007	11	0·036	55	0·005	8	0·031	47	0·0457	72	0·040	16	0·0357	56

Table X

	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Bacterial susp. 3 mg./ml.	1	1	1	1	1	1	1
Adenine stock sol.	1	1	1	1	1	1	1
Adenosine $\frac{stock}{10}$ sol.	0	0·5	0·25	0·05	0	0	0
Inosine $\frac{stock}{10}$ sol.	0	0	0	0	0·5	0·25	0·05
Water	0·5	0	0·25	0·45	0	0·25	0·45
Buffer 3 M/225	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Total vol.	3	3	3	3	3	3	3
NH_3-N in 35 min.							
mg./2 ml.	0·005	0·0178	0·0141	0·0124	0·0176	0·0139	0·0114
μl./2 ml.	8	28·5	22·6	19·8	28·2	22·2	18·3

Table XI

Concentration $= x$ (33 μl./ml.)

x Adenine	x Adenosine	x Inosine	Q_N Adenine
1	0	0	4·2
1	0·005	0	10·6
1	0	0·005	9·7
1	0·025	0	12·0
1	0	0·025	12·0
1	0·05	0	15·0
1	0	0·05	15·0
1	0·1	0	25·0
1	0·2	0	27·0

Effect of adenosine and inosine on the rate of deamination of adenine by Bact. coli *I.* Adenylic acid also has a catalytic effect on the deamination of adenine but slightly smaller than that of adenosine (Fig. 5); whilst the effect of ribose is comparatively insignificant, a concentration equivalent to that of adenine merely doubling the rate of deamination.

The effects of lower concentrations of adenosine and of inosine on the rate of deamination of adenine were then tested; the results are given in Table X, and the combined results from Tables IX and X shown in Table XI.
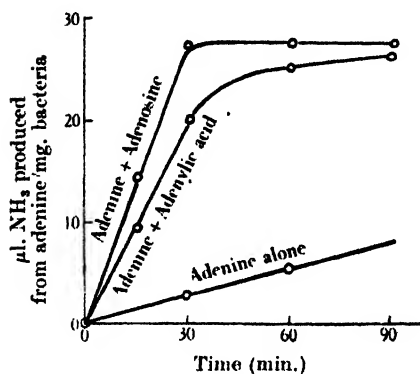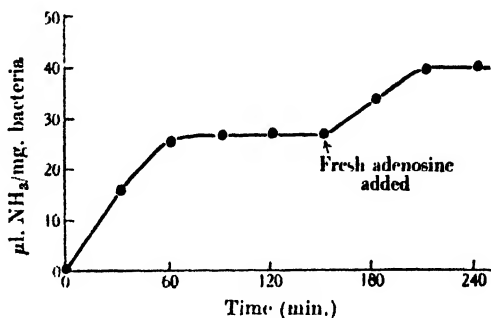
Fig. 5. The effect of adenosine and adenylic acid (3·3 μl. ml.) on the deamination of adenine (33 μl./ml.).

Fig. 6. Effect of adenosine (3·3 μl./ml.) on deamination of adenine (99 μl./ml.).

The catalytic effect of adenosine falls off with time, owing probably to its decomposition by the liberation of ribose; if fresh adenosine is added the initial rate is again restored (Fig. 6). The decreasing catalytic effect due to concentration shown in Table XI is partly accounted for in this way.

It is premature to discuss the nature of the catalytic effect of adenosine on the deamination of adenine. It is conceivable that the NH_2 group of adenine may be transferred to inosine which is then rapidly deaminated. The maximum rate of deamination of adenine in presence of adenosine is still less than that of adenosine, so evidence thus afforded is not inconsistent with this view. On the other hand Gale [1938] has recently found the rate of deamination of aspartic acid by aspartase II from the same organism increased by the addition of adenosine far beyond the rate of deamination of adenosine alone; in this case therefore the above hypothesis cannot hold and further work is necessary to arrive at the mechanism involved.

Lutwak-Mann [1936] showed that hypoxanthine was formed as end product of the action of *Bact. coli* on adenine, adenosine and adenylic acid. We have confirmed this.

Isolation of hypoxanthine from the deamination of adenine

The hypoxanthine was isolated as a silver nitrate compound [Bruhns, 1890]; 200 mg. adenosine, 100 ml. *M*/50 phosphate at *p*H 7·7 and 5 ml. bacterial suspension containing 100 mg. bacteria were incubated aerobically at 37°; after 3 hr. 2 ml. adenosine solution (66 μl./ml.) were added; the course of the reaction

was followed by NH_3 estimations; fresh adenosine was added at intervals of $1\frac{1}{2}$ hr. till $700\,\mu l.$ had been added. At the end of 24 hr. deamination had stopped having reached 70 % completion. 2 ml. $10N$ HCl was added, the whole shaken with kieselguhr and filtered; the clear filtrate was evaporated *in vacuo* to about 30 ml. and treated with excess of sodium picrate to remove the unchanged adenine, the adenine picrate was filtered on a Büchner funnel and washed. The filtrate and washings were then treated with excess ammonia and completely precipitated with silver nitrate. The precipitate (consisting of silver chloride, phosphate and a silver hypoxanthine derivative) was filtered on a Büchner funnel, washed with hot water and the residue boiled with 50 ml. HNO_3 sp. gr. 1·1; the acid was decanted off and the precipitate filtered whilst hot and re-extracted several times with HNO_3. 0·25 g. $AgNO_3$ in HNO_3 was added to the filtrate which was left 24 hr. The precipitate was filtered off and dried at 100° for 30 min. and again taken up and reprecipitated with $AgNO_3$ and dried. The analysis was as follows:

	Found %	Calculated %
C	20·52	19·68
H	1·65	1·31
N	22·8	22·95
Ag	34·78	35·49

Hypoxanthine can also be detected and estimated by the use of xanthine oxidase.

Exp. 9. 3 ml. adenylic acid stock ×2. 3 ml. phosphate $3M/450$ pH 7·7. 3 ml. bacterial suspension 3 mg./ml. 2 ml. samples were taken at intervals and 1 ml. N HCl added and the whole shaken with kieselguhr and filtered. 1·5 ml. filtrate, 0·5 ml. N NaOH and 1 ml. $M/5$ phosphate pH 7·0 were put in a Warburg manometer vessel and 0·5 ml. of a 20 % neutralized xanthine oxidase solution [Dixon & Kodama, 1926] in the side bulb. $1\,\mu g.$ hypoxanthine in presence of the xanthine oxidase preparation took up $0·24\,\mu l.$ O_2. With all substrates tested
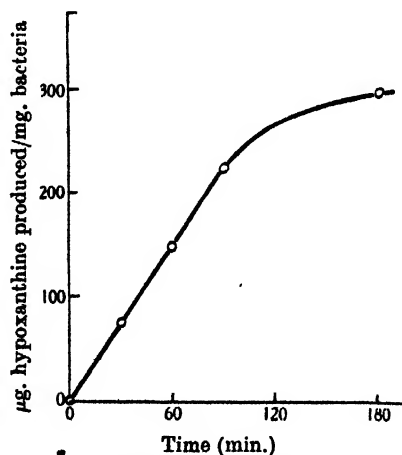
Fig. 7. Production of hypoxanthine from adenylic acid by *Bact. coli* I.

(adenylic acid, adenine and adenosine) about 70 % appeared as hypoxanthine (see Fig. 7). The bacterial suspension had a negligible xanthine oxidase activity ($Q_O = 1·5$).

APPENDIX

A MICRO-METHOD FOR THE ESTIMATION OF RIBOSE IN THE PRESENCE OF BACTERIAL SUSPENSIONS

By A. R. TRIM

Ribose may be estimated by a modification of the ceric sulphate titration method for reducing sugars previously worked out for blood sugar estimation by Giragossintz et al. [1936]. By this method the sugar is allowed to reduce ferricyanide to ferrocyanide at an alkaline reaction and then the ferrocyanide is re-oxidized with standard ceric sulphate after making strongly acid with concentrated H_2SO_4. Xylene cyanol FF is used as an indicator. The sugar does not reduce its equivalent of ferricyanide according to the stoichiometric relation represented by the equation

$$R.CHO + 2 Fe (CN)_6''' \rightarrow R.COOH + 2 Fe (CN)_6'''',$$
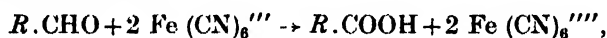
but the extent of reduction of the ferricyanide varies somewhat with the conditions of the experiment; this being the case the estimations must be made under standard conditions and a calibration curve constructed. The advantage of this method is that only the ceric sulphate need be accurately standardized; once standardized the stock solution may be kept for months without deteriorating. Provided that they are in excess, the other reagents need not be used in precise quantities, and changes during storage, so important in certain other methods, are without effect.

Before proceeding with the estimation it is necessary to remove the bacteria; trichloroacetic acid cannot be used for this purpose as during the subsequent procedure it produces substances which reduce ferricyanide. HCl is free from this disadvantage and in concentrations of $0.2 N$ to $2.0 N$ followed by filtration through kieselguhr gives a clear filtrate which on treatment with trichloroacetic gives no further precipitate.

The ribose used was supplied by Dr Georg Henning of Berlin and gave the following constants:

M.P. 85–87°; rotation $[\alpha]_D^{20} = 219$, kindly determined by Dr D. J. Bell. The analysis by Dr G. Weiler gave the following results:

C %	H %	
39·87	6·67	Found
40·0	6·58	Calculated
	Ash none.	

PROCEDURE

1 ml. bacterial suspension containing 10–250 μg. ribose was taken and 3 ml. $0.2 N$ HCl added, shaken with kieselguhr and filtered on a pleated filter paper; 2 ml. of filtrate, 2 ml. of 0·8 % potassium ferricyanide and 2 ml. of 15 % sodium carbonate were heated on a boiling water bath for 5–10 min., cooled thoroughly and one-third to one-half the total vol. 50 % H_2SO_4 added; the end point of the final titration requires this large excess of acid. To the cold acid solution a few drops of 0·2 % xylene cyanol FF were added and the solution titrated against standard ceric sulphate in N H_2SO_4; in these experiments 0·002 N ceric sulphate was used.

It was found that the reduction of the ceric sulphate for any one batch of bacteria was constant and remained unchanged when the suspension was

incubated. It was therefore possible to correct for the reduction due to the reagents and bacteria by running blank controls for each set of experiments.

A curve showing the relation between μg. ribose and ml. standard ceric sulphate was constructed over the range 0–250 μg. ribose at intervals of 50 μg. using 0·002 N ceric sulphate. The curve showed a linear relation in which 1 μg. ribose was equivalent to 0·018 ml. ceric sulphate. The concentration of the ceric sulphate is quite arbitrary, so that the amount of ribose is given by the equation:

$$x = \frac{V \times N}{0 \cdot 18 \times 0 \cdot 002} .$$

Where $x = \mu$g. ribose, $N =$ normality of the ceric sulphate and $V =$ ml. ceric sulphate used. Similar results were obtained over the range 0–100 μg. ribose at 10 μg. intervals. Table XII shows a typical set of results:

Table XII

Vol. of solution ml.	Ribose μg.	ml. ceric sulphate		ml. ceric sulphate equivalent to ribose		ml. ceric sulphate μg. ribose
		A	B	A	B	
2	0	0·47	—	—	—	—
2	0	0·47	—	—		
2	0	—	0·62	—	—	—
2	0	—	0·63	—	—	
2	50	1·31	1·46	0·86	0·84	0·0172
2	100	2·21	2·36	1·75	1·74	0·0175
2	150	3·08	3·23	2·61	2·61	0·0174
2	200	4·12	4·27	3·65	3·65	0·0183
2	250	4·90	5·05	4·43	4·43	0·0177

In both A and B bacteria were originally present and were removed before the estimations were made. In A the original solution contained 1 mg. of bacteria per ml. and in B 2 mg. per ml.

SUMMARY

1. Muscle adenylic acid is deaminated and dephosphorylated by *Bact. coli* (two strains). Dephosphorylation appears to precede deamination.

2. Deamination is completely inhibited by treatment of the washed suspension of *Bact. coli* with 1 % phenol; this treatment has no effect on dephosphorylation.

3. Deamination of adenosine is faster than that of adenylic acid (Q_N about 100 and 20 respectively with strain I) that of adenine is the slowest of the three (Q_N about 5 using strain I).

4. Adenosine increases the rate of deamination of adenine 6–7 times.

5. Adenosine (and inosine) lose ribose when acted on by *Bact. coli*. This ribose can be shown to disappear from its combination in adenosine but cannot be detected in the free state. This disappearance is shown to be due to fermentation.

6. The rate of fermentation of ribose in adenosine (or inosine) is about 10 times that in the free state.

7. The activity of strain II was less than that of strain I but the order of the activities was the same.

8. A micro-method for the estimation of ribose is appended.

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CCXXVII. ENZYME FORMATION AND POLYSACCHARIDE SYNTHESIS BY BACTERIA

III. POLYSACCHARIDES PRODUCED BY "NITROGEN-FIXING" ORGANISMS

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AND MAURICE STACEY

From the A. E. Hills Laboratories, University of Birmingham

IN a previous paper [Cooper & Preston, 1937] attention has been drawn to the question of polysaccharide formation by the nitrogen-fixing organisms, *Rhizobium radicicolum*, the root-nodule bacterium associated with the Leguminosae, and the soil organism, *Azotobacter chroococcum*. In the present paper we have confirmed the previous observations and have obtained preliminary data on the purification and properties of these interesting polysaccharides. In themselves these products are of considerable importance since they probably constitute a certain proportion of the so-called "humic substances" in soil. From our point of view they are typical examples of nitrogen-free bacterial mucilages. As already indicated, a study of the chemical and physical properties of the *Rhizobium* polysaccharides and the recognition of glucose and uronic acids among their cleavage products places them in the same class of compound as the specific polysaccharides of types II and III *Pneumococcus*. We have now shown that the *Azotobacter* polysaccharide also belongs to the same class. As a result of further study of the metabolism of these organisms, the polysaccharides are now reasonably accessible and in such quantities as to enable their chemical investigations along classical lines to be undertaken. Later it is hoped to apply the information gained to research on important questions of immunological specificity.

In this study, careful attention has been given also to the urgent problem of the possible contamination of specific polysaccharides by extraneous carbohydrate material from the media on which the organisms are cultivated. It is generally known that organisms grown on an agar medium are liable to extensive contamination with agar-polysaccharides. This danger has been particularly emphasized by Morgan [1936] in the case of the specific polysaccharide of *B. dysenteriae*. In their studies on a mucoid polysaccharide from *Streptococcus*, Heidelberger *et al.* [1937] were careful to show that no polysaccharides from the sterile broth medium were precipitated under the conditions used for isolating the bacterial polysaccharide.

In order to minimize the danger of contamination due to mechanical dislodging of agar particles we raised the agar concentration in the medium to 1·7%. In Roux bottles this gave a comparatively hard surface at 37°, and after incubation for several days the washings from these bottles were shown to contain a negligible amount of agar. Agar is constituted mainly of galactose residues so that we were particularly fortunate in being able to show that the poly-

saccharides from the nitrogen-fixing organisms were free from galactose. Of greater importance, however, was the observation that the organisms formed the polysaccharide characteristic of each on either agar or gelatin media; so it is considered that our purified products are free from extraneous carbohydrates.

Two other nitrogen-fixing organisms, *B. urea* (Beijerinck) and *B. trauffauttii*, were investigated in regard to polysaccharide formation. They grew vigorously on an agar medium but when washed off and precipitated no polysaccharides could be isolated.

Rh. radicicolum *polysaccharide*

The organism was an active culture of the clover strain kindly supplied by Dr H. Nichol of Rothamsted Experimental Station.

Extensive investigations on the growth of the organism in liquid media did not lead to a satisfactory method of polysaccharide production. The viscous material obtained was very difficult to purify and the yields were poor. On solid media, however, growth was vigorous and resulted in prolific polysaccharide formation which, moreover, was continuous over a long period. The striking advantage in the production of this type of polysaccharide on solid media may have an important application in large scale preparation of those pathogenic organisms whose antigenic properties may depend on the elaboration of complex polysaccharides.

After numerous investigations of sources of available nitrogen and carbon and of possible growth stimulators, a reliable agar medium for producing the polysaccharide was established. On a gelatin medium the same polysaccharide was produced but the growth was slower and the yield inferior.

Crude *Rh. radicicolum* polysaccharide was grossly contaminated with water-insoluble material, tenaciously held in colloidal suspension. Much of this impurity consisted of bacterial protein the greater part of which was removed by repeated filtration of a dilute aqueous solution through a pad of kieselguhr. Final traces were eliminated by exhaustive alcoholic fractionation. A typical sample of the polysaccharide was obtained as a white fibrous mass $[\alpha]_D - 17°$ in water; ash, 2%; uronic anhydride, 18%. Its hydrolysis with N H_2SO_4 was complete in about 8 hr. at $100°$. From the hydrolysate ($[\alpha]_D + 18°$) crystalline d-glucose (67% yield) and the barium salt of a uronic acid (23% yield) were isolated. The latter had $[\alpha]_D + 11°$, Ba $16\cdot0\%$, and was probably the salt of an aldobionic acid. Further investigations of this acid and the polysaccharide will be reported later.

The Azotobacter *polysaccharide*

The organism used in this study was a culture of *Azotobacter chroococcum* freshly isolated from soil and which gave a vigorous production of polysaccharide on gelatin or agar slopes. Initial investigation of its metabolism was directed towards the finding of a suitable liquid medium in which to produce the polysaccharide. Yields of purified material were so small, however, that this method was abandoned. When grown on solid gelatin or agar media results were much more consistent and the purified polysaccharides from each medium were identical. The agar medium was adopted for large scale work. This *Azotobacter* polysaccharide was purified by the method described for the *Rh. radicicolum* polysaccharide, which it resembled very closely in physical properties. Its content of bacterial protein was comparatively small and its purification was readily effected. A purified dried sample was a white asbestos-like mass, which dissolved in water forming a viscous clear solution $[\alpha]_D - 2°$; ash, 8%; uronic anhydride 4%. Hydrolysis of the polysaccharide was effected with $N/2$ H_2SO_4

at 100° in about 10 hr. From the hydrolysate ($[\alpha]_D + 26°$), crystalline d-glucose in 87 % yield and the barium salt of a uronic acid in 3 % yield were isolated. The recognition of a small percentage of a uronic acid as a constituent of the *Azotobacter* polysaccharide molecule places this polysaccharide in the same class as the type II and III *Pneumococcus* polysaccharides. Further investigations of molecular structure will be reported later.

EXPERIMENTAL

I. Rhizobium radicicolum *polysaccharide*

The organism was supplied by Dr H. Nichol (*vide supra*). It remained quite active as a gum producer on suitable medium over a long period of subculturing. The basal culture medium was essentially that previously employed [Cooper & Preston, 1937] and had the following composition:

Lucerne-root extract agar (dissolved in 500 ml. of tap water): K_2HPO_4 0·5 g., $MgSO_4$, $7H_2O$ 0·20 g., NaCl 0·20 g., $MnCl_2$ 0·01 g., $FeCl_3$ 0·01 g., $CaCO_3$ 3·0 g., sucrose 20 g., asparagine 5 g.

Powdered lucerne root (5 g.) was boiled with distilled water (500 ml.) for 1 hr., allowed to stand overnight and filtered through cotton wool. This extract was added to 500 ml. of hot basal medium in which was dissolved powdered agar (18 g.). Transference of the medium in 20 ml. portions to boiling tubes was immediately carried out. Stock cultures of the organism were maintained on 5 ml. slopes.

Investigations on liquid media. Using the above basal salt medium, additional substances in varying amounts were added in an endeavour to study the factors influencing the production of polysaccharide. In general the medium was distributed in small conical flasks, inoculated from slopes and incubated for about 10 days. The mucilaginous growth was diluted with distilled water, filtered through cotton wool and the solution run into twice its volume of alcohol. The polysaccharide which usually rose to the surface, was isolated and examined. Production of polysaccharide from media containing varying concentrations of sucrose was first studied. Observations showed that a 5 % sucrose concentration gave maximum yields. Other constituents were now varied and the following conclusions were reached:

(*a*) Addition of a maximum amount of 1 % beet molasses was sufficient to produce growth and polysaccharide formation, the latter reaching a maximum in 10 days at 30°. If molasses was employed, the medium developed a very dark colour during sterilization and the polysaccharide formed was difficult to separate from pigmented material. Initial clarification of the molasses could readily be effected by charcoal but this treatment reduced considerably its power as a growth stimulator.

(*b*) Demerara and beet sugars, alcoholic extracts of peptone or molasses were active in producing growth, whilst malt extract, maple syrup and lecithin were without effect.

(*c*) Addition of 0·02 % of caffeine stimulated polysaccharide formation but it could not replace asparagine as a nitrogen source.

For larger scale work the following medium was employed: KH_2PO_4 2 g./l., $MgSO_4$, $7H_2O$ 0·2 g./l., NaCl 0·2 g./l., $MnCl_2$ 0·01 g./l., $FeCl_3$ 0·01 g./l., $CaCO_3$ 5·0 g./l., asparagine 1·0 g./l., caffeine 0·2 g./l., sucrose 50 g./., alcoholic extract of molasses 10 ml./l.

200 ml. amounts of this medium, distributed in 500 ml. conical flasks were steamed on 3 successive days and inoculated with 1 ml. of an active 48 hr. culture

of *Rh. radicicolum* (clover variety) growing in the same medium. The flasks were incubated at 30° for 10 days. The crude polysaccharide was precipitated by pouring the viscid solution into excess of ethyl alcohol (2 vol.) containing a few drops of calcium chloride solution. The gummy fibrous mass which rose to the surface was isolated, washed with absolute alcohol and ether and dried in a vacuum. It formed a stable white asbestos-like mass. Maximum yield of crude material, 300 mg./l. of medium.

Products made on liquid medium were grossly contaminated with protein material and ash (mainly phosphate). After acid hydrolysis the polysaccharides were shown to be of variable composition and the method was abandoned.

Production of the polysaccharide on a solid gelatin medium. A medium having the following composition was prepared: KH_2PO_4 1·0 g./l., $KHPO_4$ 1·0 g./l., NaCl 0·2 g./l., $MgSO_4$, $7H_2O$ 0·2 g./l., $CaCO_3$ 5·0 g./l., $Fe_2(SO_4)_3$ 0·01 g./l., $MnCl_2$ 0·01 g./l., $CaSO_4$ 0·01 g./l., asparagine 1·0 g./l., sucrose 40·0 g./l., gelatin 150·0 g./l.

It was dispersed in 200 ml. amounts in Roux bottles, steamed on three successive days and inoculated by means of small sterile pipettes with a 48 hr. culture of the organism. After incubation at 20° for 14 days the polysaccharide was washed off with distilled water, the solution filtered through kieselguhr and purified by the method described later. Yield of crude material 50 mg./Roux bottle. It readily dissolved in water forming a viscous clear solution, $[\alpha]_D - 20°$ (*c*, 0·21). Ash, 2·0%. On hydrolysis with $N/2$ H_2SO_4 at 100° the following changes were observed: initial value $[\alpha]_D - 20°$, 45 min. $+5·0°$, 70 min. $+10°$, 120 min. $+12°$, 220 min. $+15°$ (equilibrium value).

Production of the polysaccharide on agar medium. Preliminary experiments were carried out on 20 ml. slopes in boiling tubes. After a suitable incubation period the mucilaginous growth was diluted with water, filtered through cotton wool and dropped into two volumes of alcohol. The precipitated polysaccharide was isolated in the usual manner and purified by alcoholic fractionation. It was identical with the polysaccharide grown on gelatin medium. $[\alpha]_D - 18°$ (*c*, 0·5); ash 5·0%. On hydrolysis with $N/2$ H_2SO_4 at 100° the following changes were observed: initial value $[\alpha]_D - 18°$, 100 min. $+8°$, 170 min. $+15°$, 220 min. $+18°$ (equilibrium value). No mucic acid could be obtained after nitric acid oxidation of the polysaccharide.

Control experiments were carried out: (*a*) with Roux bottles of identical agar medium but uninoculated; (*b*) using similar Roux bottles inoculated with two nitrogen-fixing organisms, *B. ureae* (Beijerinck) and *B. trauffauttii*. These bottles were incubated and treated in a manner similar to that used for isolating the *Rh. radicicolum* polysaccharide. Although the two organisms grew well on the medium in no case was any polysaccharide obtained.

From these experiments it was apparent that contamination by agar was slight, and after a series of experiments to determine optimal conditions whereby the yield per Roux bottle was increased to 0·25 g. it was considered advantageous to use the following medium for large-scale production: (dissolved in 500 ml. of water) asparagine 1·0 g., K_2HPO_4 1·0 g., $MgSO_4$, $7H_2O$ 0·2 g., NaCl 0·2 g., $MnCl_2$ 0·01 g., $FeCl_3$ 0·01 g., sucrose 40·0 g., caffeine 0·2 g., agar 17·0 g., lucerne root extract 500 ml.

The organism was grown on this medium distributed in 200 ml. amounts in Roux bottles and incubated at 25° for 10 days. The heavy growth was removed by addition of a few ml. of distilled water and rubbing gently with a glass rod. The resultant viscous solution was filtered through glass wool, and the polysaccharide isolated as usual by alcoholic precipitation.

Purification and general properties of the polysaccharide. The material, re-suspended in water, formed a viscous opalescent colloidal solution which contained appreciable amounts (60 %) of bacterial "debris" in suspension. This "debris" was mainly of a protein nature and it could not be removed by the usual centrifugal methods. Attempts to clear the solution by digestion with trypsin, pepsin or papain were partially successful, but this method was abandoned on realization that commercial preparations of these enzymes contained variable but usually significant amounts of carbohydrate material.

Intensive drying of the crude polysaccharide followed by fractional alcoholic precipitation from aqueous solution removed the bulk of suspended material which was separated in the fractions isolated at low alcohol concentrations. This method was tedious and the following "kieselguhr filtration method" was more convenient and of general application. A small amount of kieselguhr was suspended in distilled water and flooded on to a large Büchner funnel. More kieselguhr was stirred into a very dilute solution of the polysaccharide which was filtered several times under slight vacuum through the prepared filter. The filtrate was evaporated *in vacuo* to a syrup, poured into excess alcohol and the precipitated polysaccharide isolated as usual. A typical sample had the following properties: $[\alpha]_D^{18°} - 17°$ (c, 0·5); acid equivalent (after precipitation from HCl), 1000; ash, 4·6 %; moisture, 10 %; uronic anhydride, 18·0 %; nitrogen (traces).

On hydrolysis the rotation changed from $[\alpha]_D - 15°$ to $+ 22°$ (when heated with N H_2SO_4 at 80° for 2 hr.). Crystalline d-glucose ($[\alpha]_D + 52·5°$) was isolated in 67 % yield together with the barium salt of a uronic acid in 23 % yield (Ba, 16 %). This salt is being further investigated and may be an aldobionate.

Polysaccharide from the lucerne strain of Rhizobium radicicolum

According to Hopkins *et al.* [1930] the gum from the *Rh. radicicolum* (lucerne strain) contains much less glucuronic acid (4 %) than that from the clover strain which has (approx.) 20 % glucuronic acid. As we had available an improved culture medium, it was thought desirable to isolate the polysaccharide from the lucerne strain in order to ascertain whether, under conditions more advantageous to growth and metabolic activity of the organism, the polysaccharide would contain a higher percentage of uronic acid. Our observations confirmed those of Hopkins *et al.* in that "lucerne" polysaccharide from agar medium containing glucose had 4·4 % uronic anhydride and the "lucerne polysaccharide from agar medium containing sorbitol had 3·6 % uronic anhydride. Investigations are in progress to determine whether each different strain of the *Rh. radicicolum* produces a polysaccharide specific to that strain.

II. *Polysaccharide formation by* Azotobacter chroococcum

On liquid media. The organism was freshly isolated from soil at Edgbaston, Birmingham. It was particularly active as a gum-producer, for its synthetic powers did not diminish over a long period of subculturing. Investigation of polysaccharide formation in liquid media was continued and the properties of the materials isolated were compared with those of the polysaccharide grown on a solid medium. After a series of experiments during which the amounts and nature of both carbon and nitrogen sources were varied considerably, the following medium was found convenient and gave optimum yields: K_2HPO_4 0·5 g./l., $MgSO_4$, $7H_2O$ 0·2 g./l., NaCl 0·2 g./l., $CaSO_4$ 0·1 g./l., $FeCl_3$ (trace), sucrose 50·0 g./l., peptone 1·0 g./l., $CaCO_3$ 20 g./l.

Polysaccharide formation was poor when large volumes of medium were used and our results confirmed Anderson's observation [1933] that when growth did take place it was always accompanied by gum formation. Sorbitol (50 g./l.) was a good alternative to sucrose and in this case the metabolism solutions became highly viscous. The use of sorbitol minimizes any possible contamination from "laevan-forming" organisms [Cooper & Preston, 1935]. Yield of crude polysaccharide, 0·3 g./l. The products formed viscous suspensions in water which were too opaque for observation of optical rotation and very scanty amounts were obtained after purification. The medium was eventually abandoned.

On a gelatin medium. The medium used was essentially the same as that described for production of *Rh. radicicolum* polysaccharide. The method of growth, isolation and purification of the polysaccharide was also similar.

Properties of the polysaccharide. $[\alpha]_D - 2^\circ$ (c, 0·52) in water; ash, 3·1%. Hydrolysis with $N/2$ H_2SO_4 (c, 0·52), $[\alpha]_D - 2^\circ$ (initial value). 0° (30 min.), $+6^\circ$ (70 min.), $+10^\circ$ (190 min.), $+16^\circ$ (280 min.), $+20^\circ$ (355 min.), $+24^\circ$ (690 min.) (equilibrium value). A naphthoresorcinol test for uronic acid was positive.

On an agar medium. Detailed accounts of the numerous investigations carried out in order to determine the best condition of growth are unnecessary. A medium of the following composition gave reliable results: (dissolved in 1 l. of 10% yeast water) K_2HPO_4 0·8 g., KH_2PO_4 0·2 g., NaCl 0·2 g., $MgSO_4$, $7H_2O$ 0·2 g., $CaSO_4$ 0·1 g., $FeSO_4$ 0·01 g., $CaCO_3$ 5·0 g., sucrose 50 g., agar 17 g.

The medium was distributed in 200 ml. portions in Roux bottles, sterilized by steaming on three successive days and inoculated (using sterile pipettes) from an emulsion of a 48 hr. fluid culture. After incubation for 10 days at 25° the white viscous growth was washed off with a small amount of distilled water and the solution filtered through cotton wool into excess alcohol. The polysaccharide was readily precipitated in the form of a white fibrous mass which in appearance closely resembled the *Rh. radicicolum* polysaccharide, although the latter usually contained considerably larger quantities of bacterial protein. Purification of the polysaccharide was readily effected by filtration through kieselguhr in the manner previously described. The polysaccharide showed $[\alpha]_D - 2^\circ$ (c, 0·52). It dissolved rapidly in water to give a viscid clear solution which was neutral. Ash, 8·3%; uronic anhydride, 4%. Hydrolysis was carried out at 100° with $N/2$ H_2SO_4 and the following rotational changes were observed: initial value $[\alpha]_D - 6^\circ$ (c, 1·2), 60 min. -2°, 96 min. $+2^\circ$, 138 min. $+5^\circ$, 168 min. $+7^\circ$, 210 min. $+10^\circ$, 330 min. $+17^\circ$, 510 min. $+25^\circ$, 570 min. $+26^\circ$ (equilibrium value). From the hydrolysis solution crystalline glucose, $[\alpha]_D^{20^\circ} +52·3^\circ$ (c, 1·0), was isolated in 87% yield together with a small amount of the barium salt of a uronic acid (3% yield) which gave a positive napthoresorcinol test. Tests for ketose or pentose were negative and no mucic acid could be isolated from oxidation of the hydrolysate.

SUMMARY

Suitable media for large scale preparation of the polysaccharides from *Rhizobium radicicolum* (clover strain) and from *Azotobacter chroococcum* have been described. The polysaccharides have been purified and their hydrolysis products examined. This *Azotobacter* polysaccharide contains glucose units (about 90%) and a small amount of a uronic acid residue (3–4%). In this respect and in physical properties the polysaccharide resembles that from *Rh. radicicolum* and both probably belong to the same class as the specific polysaccharides of *Pneumococcus* types II and III.

The authors wish to express their thanks to Prof. W. N. Haworth and Dr S. Peat for their interest in the work, and to Dr H. Nichol of Rothamsted Experimental Station for kindly supplying cultures.

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CCXXVIII. THE ISOLATION OF ANDROSTERONE AND *TRANS*DEHYDROANDROSTERONE FROM THE URINE OF NORMAL WOMEN

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IT was first reported by Womack & Koch [1932] that the urine of normal women yielded quantities of androgenic material which, as measured by the capon test, were of the same order as that yielded by the urine of normal men. This fact has been confirmed repeatedly [cf. Siebke, 1934; Dingemanse *et al.* 1935; 1937; Simpson *et al.* 1936; Callow, 1936; Koch, 1936; Gallagher *et al.* 1937; Callow, 1938; Callow *et al.* 1938] and, with the improved methods of extraction which have been used during the last two years, it is generally recognized that, although the average amount of urinary androgen is somewhat lower for women than for men, the limits of normal variation for the two sexes overlap. Nevertheless, there has been no report of attempts to identify chemically the androgenic compounds in the urine of normal, non-pregnant women, parallel with the work by Butenandt and his collaborators [1934; 1935; 1937], which established that androsterone and *trans*dehydroandrosterone occurred in the urine of men, and indicated the presence of *iso*androsterone and of *epi*aetiocholanediol.

Work carried out in this laboratory [Callow *et al.* 1938] has shown that in respect of the ratio of methyleneketone, or "sterone" content (estimated colorimetrically) to comb-growth activity, no distinction can be made between urine extracts from men or women, but this rather vague evidence required amplification by more precise chemical analysis. With this end in view we undertook the more detailed fractionation of extracts of urine from both males and females. The concentration of androgenic activity in the neutral ketonic fraction of men's urine obtained by the use of Girard's reagent has been described in a previous paper [Callow *et al.* 1938]. When the ketonic resin was distilled at low pressure, volatile fractions were obtained, from the first of which the oxime of androsterone could be separated in the pure state in a yield of 0·2 mg. per l. of original urine. Parallel with these experiments, a concentrate from women's urine was put through the same process. There was a striking similarity in the behaviour of the two types of extract in the whole course of the fractionation, and this culminated in the isolation from the women's urine concentrate of androsterone oxime in a yield of 0·44 mg. per l. of original urine. The identity of the compound was confirmed by hydrolysis to androsterone and conversion of the latter into the benzoate. From a second fraction, distilling at a higher temperature, *trans*dehydroandrosterone was isolated in small amount as the characteristic benzoate.

EXPERIMENTAL

Collection and extraction of urine. Urine was collected from seven individual women patients in Middlesex Hospital. Their ages ranged from 20 to 35 years, and none had any obvious sexual dysfunction. Toluene was used as preservative,

and the collections from periods of 6–7 days, having pH values from 5·5 to 7, were hydrolysed and extracted as soon as they were available, using the method described by one of us [Callow, 1936]. The neutral fractions were made up in alcoholic solution and, after removal of sufficient material for colorimetric and capon assays, the solutions were combined and evaporated to dryness. The extract from 75 l. of urine or 76 days' output obtained in this way was dissolved in 150 ml. of hot methanol, kept overnight and filtered from a small amount of insoluble material. Evaporation of the filtrate yielded a residue weighing 2·59 g. A colorimetric assay by the modified Zimmermann method [Callow et al. 1938] gave a value of 8·2 mg. of methyleneketone or "sterone" per litre, i.e. the chromogenic equivalent of 0·615 g. of 17-ketosteroids in the whole sample.

Separation of ketonic fraction. The methanol-soluble fraction of the extract from 32 l. of urine, wt. 1·13 g., was dissolved in 20 ml. of glacial acetic acid, 2 g. of Girard's reagent T added, and the mixture heated on the water bath for 30 min. After cooling, it was poured into 400–500 ml. of ice and water containing enough Na_2CO_3 to neutralize, to phenolphthalein, 18 ml. of acetic acid. The mixture was extracted 7 times with 100 ml. lots of ether to remove the non-ketonic fraction. The combined ether extracts were washed once with water, which was added to the aqueous ketonic layer. The latter was acidified with 10 % of its volume of conc. HCl, warmed gently and kept standing for 1·5 hr. It was then extracted 5 times with 100 ml. lots of ether; the extract was washed with water, 0·5 N Na_2CO_3 and again with water, dried over Na_2SO_4 and evaporated. The residue weighed 423 mg. The ethereal solution of the non-ketonic fraction was washed, dried and evaporated. Wt. of residue, 487 mg. The ketonic fraction was dissolved in acetic acid and treated again with Girard's reagent. A ketonic fraction weighing 292·7 mg. was obtained. Colorimetry indicated a content of 75 % of "sterone".

Isolation of androsterone. The ketonic material, in alcoholic solution, was transferred to the vacuum still and evaporated to dryness. The still used consisted of a vertical, cylindrical tube with rounded bottom, of 4·5 cm. internal diameter, with a concentric water-cooled condenser of 3·5 cm. external diameter reaching to within 0·5 cm. of the bottom of the outer tube. Distillation was carried out at 0·0009 mm. and a bath temperature of 90–95° for 3 hr. 77·4 mg. of an almost colourless resin distilled. A colorimetric estimation gave a value of 80 % of "sterone".

72 mg. of the distillate, 40 mg. of hydroxylamine hydrochloride and 40 mg. of anhydrous sodium acetate were dissolved in about 10 ml. of alcohol and boiled under reflux for 5½ hr. The solution was filtered to remove NaCl, and evaporated to a volume of 2–3 ml. A few drops of water were added, and an oil separated, which crystallized on scratching. Next day the solid was collected, washed rapidly with a little cold 50 % aqueous alcohol and dried: yield, 21·6 mg., M.P. 149–194°.

After three recrystallizations from acetone, a product was obtained crystallizing in well-formed rhombic plates, M.P. 208–213·5° after softening at 204° (Kofler apparatus; see note below). The M.P. was unchanged by admixture with an authentic specimen of androsterone oxime (M.P. 210–211°).

14 mg. of once recrystallized oxime, M.P. 203–206°, were dissolved in 2 ml. alcohol, 1 ml. of 3N H_2SO_4 added and the mixture boiled under reflux for 3 hr. The crude ketone, precipitated by water, weighed 3 mg.; M.P. 168·5–175°. It crystallized from methanol in thin plates, M.P. 178–181°, after subliming to needles. A mixture with an authentic specimen of androsterone (M.P. 184–185°) melted at 178–181·5°.

About 2·5 mg. of crude androsterone (recrystallized product and material obtained by evaporation of the mother liquors above) were dissolved in 0·5 ml. of pyridine, and 2 drops of benzoyl chloride added. The mixture was warmed on the water bath for 5 min., cooled and treated with water drop by drop. An oil separated, which was extracted with ether. The extract was washed with water, dilute HCl, $NaHCO_3$ solution and again with water, dried over Na_2SO_4 and evaporated. The residue was crystallized from 2 drops of methanol. The product, M.P. 166·5–171° (soft at 166°), was again recrystallized from methanol. It then had M.P. 172–175° (soft at 169°), and a mixture with an authentic specimen of androsterone benzoate (M.P. 170–174°) melted at 170–174·5° (soft at 168°).

Separation of trans*dehydroandrosterone benzoate.* 192 mg. of ketonic fraction (from 20 l. of urine) were distilled as described above at 90–95°/0·002 mm. for 2 hr. After removal of the distillate, the still was evacuated to 0·0017 mm., the oil bath heated slowly to 145° and then kept at 145–150° for 1½ hr. 84 mg. of distillate were obtained. Colorimetric assay indicated a content of 87% of "sterone". A comparative experiment showed that *trans*dehydroandrosterone slowly sublimed between 120 and 150° under the above conditions.

The distillate was dissolved in about 2 ml. of dry pyridine, treated with 3 drops of benzoyl chloride and the mixture warmed for 5 min. It was then cooled and treated with water. An oil separated which was well washed with water and dissolved in about 2 ml. of methanol. After leaving overnight in the refrigerator, 1–2 mg. of a crystalline solid separated, M.P. 223·5–243·5°. After recrystallization once from methanol and once from ethyl acetate, the M.P. was 246–248° (softening 245°). A mixture with an authentic specimen of *trans*dehydroandrosterone benzoate (M.P. 245·5–250°) melted at 245–248·5°. A further crop of crystals separated from the original methanol mother liquors which after recrystallization melted at 238–248° (mixture with *trans*dehydroandrosterone benzoate, M.P. 237–252°).

All the melting points recorded were observed on a microscope slide on an electrically heated stage, using Kofler's micro-melting point apparatus. The temperatures recorded are those of the first appearance of liquid and of complete fusion.

DISCUSSION

It is abundantly clear that complete chemical analysis of extracts of normal urine into their steroid components is a difficult matter. The processes of partition between solvents, distillation and crystallization, not ideal for quantitative work at the best of times, are not well adapted to the separation of closely related steroids. Our yields of androsterone amounted to about the equivalent of 0·2 and 0·4 mg. per litre from urines of men and women, respectively. The higher yield in the latter case is to be attributed to practice in the manipulation, and there are clear indications that extracts of urine from normal men or women contain similar amounts. We do not feel, however, that it is possible to estimate the amounts originally present more accurately than Butenandt and his collaborators [1935] did when they concluded that the amounts of androsterone and *trans*dehydroandrosterone actually present in extracts of men's urine were at least twice those separated in the pure state. With the available methods of separation we are, in fact, very far from the stage of being able to account, by isolation of recognizable derivatives, either for the whole androgenic activity, or for the colorimetrically determined methylene-ketone content of normal urine extracts.

The importance of the present work lies in its demonstration that androsterone and *trans*dehydroandrosterone are not specific products of the male

organism. It is possible, however, that the excretion of identical androstane derivatives by the two sexes is simply due to these products being common degradation products, particularly adapted for excretion, of original secretions which, although different, are closely related in chemical structure. The alternative assumption of actual identity of the original secretions in the two sexes is more speculative, and could only apply to a part of the endocrine steroids, since the nature of only a fraction of the excretory transformation products is established. It has, however, some plausibility as regards products from the suprarenal glands, for evidence from suprarenal tumour cases leads to the belief that *trans*dehydroandrosterone at least is metabolically derived from the family of steroid compounds present in the suprarenal glands, and that its excretion is not directly connected with gonadal function (cf. the discussion by Callow [1938]). The degree of direct responsibility of the gonads for any part of the urinary excretion of androstane derivatives remains to be investigated; at present there is no clue to products which can be recognized as derived from the testis or the ovary, and any characteristic difference in the androstane derivatives excreted by the two sexes still eludes detection. It is hoped that more light on these problems will be obtained as chemical and physical methods are developed and applied to the more detailed analysis of urines from abnormal clinical types of patient. Work along these lines is now in progress.

SUMMARY

Androsterone and *trans*dehydroandrosterone have been isolated from extracts of the urine of normal women, in a yield comparable with that from normal men's urine.

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CCXXIX. ELIMINATION OF AMINES IN MAN

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THE amine oxidase of mammalian tissues has been shown to oxidize adrenaline and a number of other amines to the corresponding aldehydes [Richter, 1937; Blaschko *et al.* 1937]. Of the 67 amines hitherto tested those of the general type $R.CH_2NH_2$ were readily oxidized, but amines of the type $RR'CH.NH_2$ showed no appreciable oxidation. The latter group includes the amines of the ephedrine series which are of particular interest owing to their selective action on certain parts of the nervous system and experiments were therefore carried out to determine what happens to them in the animal body and how they are eliminated.

Indications have been recorded in the literature that the elimination of amines may in some cases take a different course in laboratory animals from that followed in man. Slotta & Müller [1936] found for example that mescaline is oxidized in dogs and rabbits to the corresponding acid which they isolated from the urine, but they were unable to detect this acid in the urine in man.

In the present experiments a series of amines were given by mouth or intravenously to male adults of 60–75 kg. and believed to be normal. In agreement with the previous work with the isolated amine oxidase, amines of the type $R.CH_2NH_2$ were rapidly destroyed *in vivo* while amines of the ephedrine series were found to be excreted in the urine unchanged.

With benzedrine and methylisomyn the non-toxic dose is of the order of 10 or 20 mg., and since the excretion extended over 2 days the resulting concentrations in the urine were too small to be estimated by the ordinary methods available: a sensitive micro-method for estimating amines was therefore worked out in order to follow the elimination quantitatively.

Micro-estimation of amines

The method depends on the fact that picric acid gives little colour while amine picrates are strongly coloured in 50 % chloroform-toluene or chloroform-light petroleum solution: it is generally applicable to the higher amines and alkaloids which are extracted from aqueous solution by organic solvents [Richter, 1938]. 60 ml. of urine in a 250 ml. separating funnel were made alkaline with 4 ml. $2N$ NaOH and shaken with 6 ml. toluene, using a rotating motion to avoid froth formation. The toluene layer was run into a centrifuge cup, centrifuged for 5 min., cleared by stirring with a glass rod and centrifuged again. 0·1 ml. of 2 % picric acid in chloroform was added to 3 ml. of the toluene solution and 3 ml. of chloroform in a dry test tube and the mixture was kept for 12 hr. The clear yellow solution of the amine picrate was then compared colorimetrically with solutions prepared in a similar manner from standard amine solutions.

By this method concentrations as low as 0·5 μg./ml. of β-phenylethylamine could easily be estimated. The urines were collected in bottles containing toluene to prevent amine formation by bacterial action. Normal urines were generally found to contain traces of amines coming apparently from the food (Lintzel [1934] observed an increased excretion of amines after feeding fish): but by giving a diet consisting chiefly of bread and butter the normal amine content of

the urine could be reduced to a value corresponding to 0–0·5 μg./ml. of phenylethylamine. Of the basic substances commonly present in urine which might be expected to interfere ammonia, methylamine, trimethylamine, trimethylamine oxide, indole and skatole at concentrations of 20 μg./ml. gave less colour than 0·5 μg./ml. of β-phenylethylamine and could be neglected. Bain [1914] claimed to have isolated *iso*amylamine from urine and that, if present, would interfere; but Guggenheim & Löffler [1915] were unable to confirm the presence of *iso*amylamine in normal urine and concluded that it is formed only by subsequent bacterial action on standing. No interference by *iso*amylamine was observed under the conditions of the present experiments.

Ephedrine. $C_6H_5CHOH.CH(CH_3)NH(CH_3)$

(a) *Identification.* Urine was collected for 12 hr. after feeding ephedrine hydrochloride (104 mg. base). The urine was made alkaline with NaOH, extracted with ether and the ethereal extract shaken with a small volume of $N/10$ HCl. On evaporation and recrystallization of the residue a hydrochloride was obtained which crystallized in colourless prisms showing parallel extinction and strong interference in polarized light, crystal edge angle 130° and M.P. 215–217°. Ephedrine hydrochloride crystals showed similar optical properties, crystal edge angle 131° and M.P. 217–219°. The mixture melted at 216–218°.

(b) *Rate of excretion.* Urine was collected every 3 to 4 hr. before and after giving ephedrine hydrochloride by mouth and the ephedrine in the urine was estimated by the micro-method described. The rate of excretion was maximal
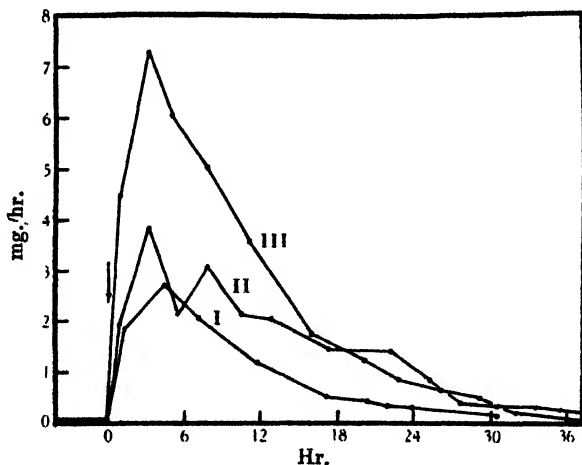
Fig. 1. Excretion of ephedrine in the urine. I, 26 mg. base; II, 52 mg. base; III, 104 mg. base. Given as the hydrochloride by mouth.

(115 μg./ml.) at about 4 hr. after administration, but with a large dose of 104 mg. a slow excretion at the rate of 0·25 mg./hr. could still be detected after 36 hr. The figures obtained for the fractions excreted at different times after administration were as follows:

Ephedrine (mg. base)	Time after administration		
	12 hr. %	24 hr. %	48 hr. %
26	77	97	97
52	59	92	99
104	59	77	82

These figures show that ephedrine is not appreciably oxidized in the body, but approximately 100 % may be excreted unchanged in the urine. The lower value of 82 % excretion obtained with 104 mg. ephedrine suggests that other methods of elimination, as in the sweat or faeces, may become significant when large amounts are given.

Benzedrine. $C_6H_5CH_2CH(CH_3)NH_2$

(a) *Identification.* Urine collected for 12 hr. after giving benzedrine sulphate (14·7 mg. base) by mouth was made alkaline with NaOH and extracted with toluene. The toluene solution was shaken with 10 ml. 1 % HCl and the aqueous solution evaporated to dryness. The residue was transferred to a Conway dish and the picrate, picrolonate and 2 : 4-dinitronaphthol derivative were prepared by allowing the base to volatilize in the presence of traces of the reagents on microscope coverslips [Richter, 1937]. The picrate crystallized in yellow prisms showing parallel extinction and strong interference colours in polarized light and crystal edge angles 107, 124 and 134°. Benzedrine picrate crystallized in similar habit and gave similar optical properties and crystal edge angles 106, 124 and 133°. The picrolonates and dinitronaphthol derivatives also agreed in crystal habit and optical properties: the pleochroism of the picrolonates was very characteristic.

(b) *Rate of excretion.* 20 mg. benzedrine sulphate (14·7 mg. base) were given by mouth and the urinary amines were estimated before and after administration, using light petroleum for the extraction. The rate of excretion was slower than for ephedrine and it was confirmed in a number of experiments that benzedrine could still be detected in the urine for 36 hr. after administration. With 14·7 mg. base the fraction excreted in the urine in 24 hr. was 40 % and in 48 hr. 70 %.
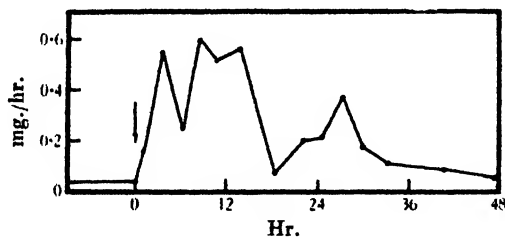
Fig. 2. Excretion of benzedrine (14·7 mg. base).

These figures make it unlikely that benzedrine is oxidized to any appreciable extent in the tissues. If the blood volume is taken as 7 l. the concentration in the blood for this dose would be at the most 2·1 μg./ml. and is probably much lower than this since the base is lipoid-soluble and is known to enter the nervous system. The concentration of benzedrine found in the urine was 10–13 μg./ml. a few hours after administration so that the kidneys must effect considerable concentration of the amine.

Methylisomyn. $C_6H_5CH_2CH(CH_3)NH(CH_3)$

Methylisomyn hydrochloride (8 mg. base) was given by mouth. As for benzedrine the rate of excretion was slow, being significant at 48 hr. after administration, and the recovery in the urine was 36 % in 24 hr. and 56 % in

48 hr. The maximal concentration found in the urine was $5\,\mu g./ml.$ which again shows a considerable concentration by the kidneys such as was also found with ephedrine and benzedrine.
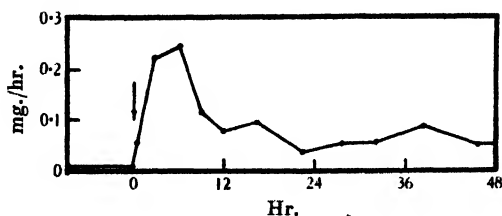
Fig. 3. Excretion of methylisomyn (8 mg. base).

Amines of the type $R.CH_2NH_2$

The following amines were given by mouth in the form of the hydrochlorides: *iso*amylamine (100 mg. base), benzylamine (160 mg. base), β-hydroxy-β-phenyl-ethylamine (50 mg. base) and β-phenylethylamine (300 mg. base). Although the experimental method was sufficiently sensitive to detect $1\,\mu g./ml.$ of amine no increased amine excretion was observed after administration.

Attempts were then made to show the rate of elimination of β-phenylethyl-amine by looking for the corresponding phenylacetic acid in the urine: Guggenheim & Löffler [1915] showed that phenylacetic acid is formed from β-phenylethylamine in the rabbit.

Identification of phenylacetic acid. Urine voided after feeding β-phenylethyl-amine hydrochloride (300 mg. base) was heated for 3 hr. at 100° with 0·1 vol. of conc. H_2SO_4 to decompose phenylacetylglutamine [Thierfelder & Sherwin, 1914]. The acids obtained by extracting with toluene were separated by fractional crystallization and consisted mainly of benzoic acid and an acid which crystallized from water in triangular or hexagonal plates giving crystal edge angle 128° and having a characteristic smell. Phenylacetic acid crystallized in similar habit and gave the edge angle 128° and the same smell.
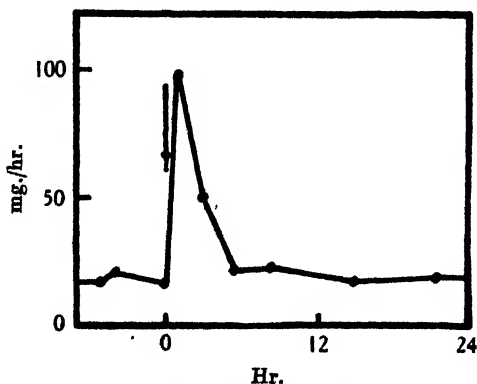
Fig. 4. Excretion of phenylacetic acid after taking β-phenylethylamine (300 mg. base) by mouth.

Rate of excretion of phenylacetic acid. The normal excretion of benzoic acid was reduced to a minimum by keeping on a diet free from fruit and vegetables.

Urine specimens collected before and after feeding β-phenylethylamine hydrochloride (300 mg. base) were heated for 3 hr. at 100° with 0·1 vol. conc. H_2SO_4. 100 ml. samples of the urine were then shaken with 10 ml. of toluene and the toluene-soluble acids were titrated with $N/10$ NaOH using thymol blue. A partition curve was then prepared by treating standard solutions of phenylacetic acid in a similar manner and so the concentration of toluene-soluble acid in the urine, taken as phenylacetic acid, was estimated. No information is available as to the rate of conjugation of phenylacetic acid with glutamine to form the phenylacetylglutamine which is excreted, but in the present experiments the increased excretion of toluene-soluble acid corresponded to 62 % of the theoretical for 300 mg. β-phenylethylamine in $4\frac{1}{2}$ hr.: the oxidation of the amine must therefore be comparatively rapid.

Mescaline. $(OCH_3)_3C_6H_2CH_2CH_2NH_2$
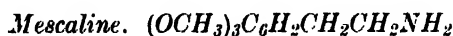
(a) *Identification*. Mescaline hydrochloride (191 mg. base) was given intravenously and the urine was made alkaline and extracted with toluene. The toluene solution was concentrated and treated with a solution of picric acid in chloroform. The picrate which crystallized out on standing formed diamond-shaped prisms giving an extinction angle of 10° in polarized light, crystal edge angle 141° and M.P. 221–223°. Mescaline picrate crystallized in similar habit and gave similar optical properties, extinction angle 10°, edge angle 142° and M.P. 221–223°. The mixture showed no depression of M.P. Mescaline was also identified in the same way in the urine after giving by mouth.

(b) *Rate of excretion*. When mescaline hydrochloride (191 mg. base) was given intravenously the fractional excretion in the urine was 52 % in 24 hr. and with the same dose given by mouth 58 % in 24 hr. The main bulk of the mescaline
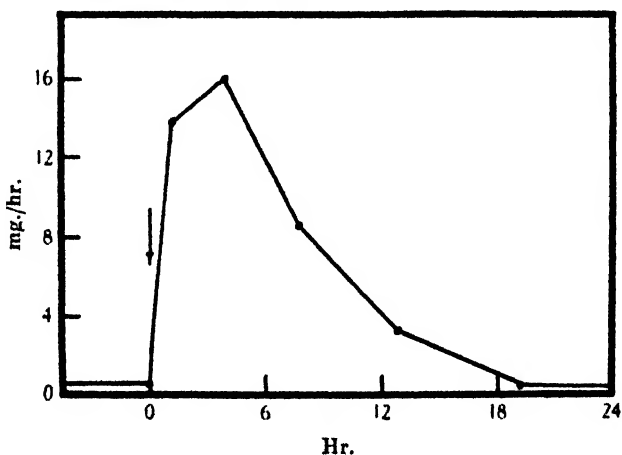
Fig. 5. Excretion of mescaline after taking 191 mg. base as the hydrochloride by mouth.

is clearly excreted unchanged, but the amount found in the urine falls considerably short of 100 % which suggests that the body may have a certain limited capacity for oxidizing mescaline or eliminating it in some other way.

DISCUSSION

The work of Ewins & Laidlaw [1910] and Guggenheim & Löffler [1915] led to the general conclusion that amines are rapidly eliminated by oxidation in the animal body. On the other hand there is pharmacological evidence that amines may in some cases remain active in the body for a considerable length of time. The action of ephedrine for example is known to continue for many hours and the effects of benzedrine have been observed to persist for more than a day [Guttmann & Sargant, 1937]. It has always remained doubtful, however, how far the various effects observed were due to the direct action of the amines and how far they were after-effects due to tissue damage caused by the amines: this could be decided only by accurate information as to the rate of elimination.

The present experiments *in vivo* confirm the previous observations on the significance of the amine oxidase since those amines which are oxidized by the enzyme (type $R.CH_2NH_2$) were also destroyed in the body, while amines of the ephedrine series (type $RR'CH.NH_2$) which are not oxidized by the enzyme were slowly excreted in the urine from which they could be recovered unchanged. The pharmacological data as to the persistent action of amines of the ephedrine series can therefore be explained in terms of the specificity of the amine oxidase.

With amines that are oxidized in the body the efficiency of the inactivation *in vivo* is shown in the case of β-phenylethylamine by the promptness with which phenylacetic acid appears in the urine and by the fact that even with doses of 300 mg. no amine excretion could be detected in the urine.

Mescaline, which is a phenylethylamine derivative, might be expected to be oxidized by the amine oxidase, but Blaschko *et al.* [1937] found little oxidation of mescaline by preparations of guinea-pig amine oxidase and it would appear that the bulky methoxyl groups modify the molecule so that it is able to resist oxidation. Slotta & Müller [1936] found that mescaline is completely oxidized *in vivo* in the rabbit and dog, but Bernheim & Bernheim [1938] concluded that some factor other than the amine oxidase is responsible for the oxidation. Whatever this factor may be it is clearly deficient in man and the case of mescaline illustrates the fact that with drugs of this type the behaviour in one kind of animal may be quite different from that in another.

SUMMARY

1. A sensitive micro-method for estimating amines has been used for studying the elimination of amines in man.

2. Amines of the ephedrine series and also mescaline are excreted unchanged and were isolated from the urine in the form of crystalline derivatives.

3. Amines of the general type $R.CH_2NH_2$ are rapidly oxidized in man.

4. The rates of excretion of ephedrine, benzedrine, methylisomyn and mescaline have been measured.

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CCXXX. THE USE OF COMPOUNDS RELATED TO *p*-AMINOBENZENESULPHONAMIDE IN THE TREATMENT OF CERTAIN INFECTIONS IN MICE

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IT is now well established that *p*-aminobenzenesulphonamide has some effect on septicaemias in mice other than those caused by the haemolytic *Streptococcus* [Buttle *et al.* 1936; Proom, 1937; Buttle *et al.* 1937 etc.], and further that various related compounds exert chemotherapeutic action in certain mouse infections. However, in no case has it been shown that the *in vivo* effects run parallel with the *in vitro* results, and the mode of action is still very obscure.

The object of the present investigation has been to compare the chemotherapeutic actions of a number of selected compounds on mice infected with various pathogenic bacteria. The organisms chosen were the *Streptococcus*, the *Staphylococcus*, the *Pneumococcus* and *Bacillus aertrycke*. At the same time experiments were carried out to determine what action, if any, the compounds exerted on the growth of these bacteria *in vitro* in order to ascertain whether the action *in vitro* could in any way be correlated with the chemotherapeutic activity as exhibited in animal experiments.

In vivo *experiments*

A. *Infection of animals.* Using the mouse as the experimental animal approximately 100 M.L.D. of the infecting organism were injected intraperitoneally. The inoculum was obtained from an 18-hr. slope culture which was emulsified and made up to the required dilution with either sterile saline or 5% mucin. Some organisms were not sufficiently virulent to kill mice when suspended in saline, and so a method involving the use of mucin similar to that of Miller & Castles [1936] was used to diminish the minimum fatal dose of these bacteria. 1 ml. of the bacterial dilution was injected intraperitoneally into mice of 20–25 g. weight.

B. *Chemotherapeutic treatment.* The various compounds under investigation were first tested for their toxicity when administered to normal mice orally through a feeding tube. Whenever possible the compound was brought into solution in a suitable solvent, but otherwise it was given in the form of a suspension in gum acacia. The doses given to the infected mice varied according to the toxicity but in each case the drug was fed immediately after the injection of organisms and at intervals thereafter. The frequency of feeding varied according to the reaction of the animal to the organism and to the drug.

The non-toxic dose employed for the majority of compounds was 10 mg. administered by mouth twice daily for 2–3 days.

Results of animal experiments. The results obtained from experiments with a series of compounds are shown in Table I. In the case of *p*-aminobenzenesulphonamide, with the *Streptococcus* as the infecting organism, the usual clear-cut

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Table I. In vivo experiments

100 M.L.D. of infecting organisms injected intraperitoneally into mice of 20–25 g.
10 mg. therapeutic agent fed immediately, after 5 hr. and twice daily for 2–3 days.

Compound	Formula	Oral toxicity for 20 g. mouse mg.	Solvent	Therapeutic activity			
				Strepto-coccus	Pneumo-coccus	Staphylo-coccus	Bacillus aertrycke
p-Aminobenzenesulphonamide	NH_2—◯—SO_2NH_2	80	Water	+ + +	+ to ±	±	±
p-Hydroxybenzenesulphonamide	HO—◯—SO_2NH_2	30–40	Water	± to 0	±	±	0
3-Nitro-4-hydroxybenzene-sulphonamide	HO—◯(NO_2)—SO_2NH_2	>100	Sodium bi-carbonate	+ +	±	0	0
3-Amino-4-hydroxybenzene-sulphonamide	HO—◯(NH_2)—SO_2NH_3	80	Suspension in gum acacia	±	±	±	0
p-Aminobenzenesulphonamide coupled with p-hydroxyben-zenesulphonamide	NH_2SO_2—◯—N:N—◯(OH)—SO_2NH_2	20	Water	±	±	0	0
p-Aminobenzenesulphonamide coupled with 8-hydroxyquino-line		20	Suspension in gum acacia	± to 0	0	±	0
p-Aminobenzenesulphonamide coupled with 8-hydroxyquino-linemethosulphate		60	Water	± to 0	0	±	0
4:4′-Dinitrodiphenylsulphide	NO_2—◯—S—◯—NO_2	>100	Suspension in gum acacia	0	±	0	0
4:4′-Diaminodiphenylsulphide	NH_2—◯—S—◯—NH_2	About 60	Lactic acid	±	±	0	0
4:4′-Diacetyldiaminodiphenyl-sulphide	CH_3CONH—◯—S—◯—$NHCOCH_3$	>100	Suspension in gum acacia	±	±	+ to ±	0

+ + + 100% recovery, + + 75% recovery, + 25–30% recovery, ± death delayed, 0 no effect.

evidence of chemotherapeutic activity was obtained, 100 % of the animals infected recovering completely within 3–4 days. With the other compounds the best result was obtained with 3-nitro-4-hydroxybenzenesulphonamide when, in a series of haemolytic streptococcal experiments, 75% of the treated animals survived indefinitely. This compound also showed slight action against the pneumococcal infection of mice but there was no effect on the *Staphylococcus* or on *Bacillus aertrycke*. As the toxicity of this compound is less than that of *p*-aminobenzenesulphonamide its use as a therapeutic agent is worthy of consideration.

Of the other results the most important is the antistaphylococcal action of diacetyldiaminodiphenylsulphide.

In vitro experiments

Technique. When possible, solutions of the compounds were prepared: otherwise fine suspensions were employed. In every case the *p*H was adjusted to 7·6. Dilutions were made in broth or serum broth according to the organism to be tested. These broth tubes were inoculated with 0·1 ml. of a thick bacterial suspension obtained by emulsifying an 18-hr. slope culture of the organism, and incubated at 37° for 18 hr. In order to determine both the bacteriostatic and the bactericidal activities of the compound, subcultures from the broth tubes were made on blood-agar plates after 3, 6 and 18 hr. incubation, and from the growth readings of these plates the activity of the drug was ascertained. The results obtained with the series of compounds used in the *in vivo* experiments is shown in Table II.

Table II. In vitro experiments

Each papain broth tube was inoculated with 0·1 ml. of a thick suspension of organisms, *p*H 7·6. *r*H 19–20. Subcultures were made after 3, 6 and 18 hr., and from these readings the bacteriostatic and bactericidal activities were estimated.

Compound	Bactericidal and bacteriostatic activities			
	Strepto-coccus	*Pneumo-coccus*	*Staphylo-coccus*	*B. aertrycke*
p-Aminobenzenesulphonamide	+ + ↑	↑ + →	0	- to 0
p-Hydroxybenzenesulphonamide	+ – ·	· ↑ ·	0	0
3-Nitro-4-hydroxybenzenesulphonamide	↑ + + +	↑ + ·	0	0
3-Amino-4-hydroxybenzenesulphonamide	↑ ±	+ – ↑	0	0
p-Aminobenzenesulphonamide coupled with *p*-hydroxybenzenesulphonamide	+ +	±	· to 0	0
p-Aminobenzenesulphonamide coupled with 8-hydroxyquinoline	+ + to +	+ → - ±	·	±
p-Aminobenzenesulphonamide coupled with 8-hydroxyquinolinemethosulphate	↑ + to +	↑ + + +	±	±
4:4′-Dinitrodiphenylsulphide	+ +	+ +	±	0
4:4′-Diaminodiphenylsulphide	± + to ↑	+ +	±	0
4:4′-Diacetyldiaminodiphenylsulphide	+ + to +	+ ··	±	± to ±

+ + + + 　Bacteriostatic at 1 in 10,000, bactericidal at 1 in 1000.
+ + + 　　　　,,　　　 1 in 10,000,　　 ,,　　 1 in 100.
± + 　　　　,,　　　 1 in 1000,　　　 ,,　　 1 in 10.
+ 　　　　,,　　　 1 in 100.
± 　　　　,,　　　 1 in 10.
0 No effect.

Results of in vitro *experiments.* The bactericidal action of these compounds on the *Pneumococcus* is greater than is to be expected from the results of animal experiments. Though this is partly to be explained by the low viability of the *Pneumococcus* as compared with other organisms, the difference between the

antipneumococcal activities of *p*-hydroxybenzenesulphonamide and the quinoline derivatives in the test tube and in the living animal requires further investigation. Although many compounds, as is shown in Table II, are bactericidal to the *Pneumococcus*, yet in no case has it been possible to cure mice with a pneumococcal septicaemia.

The results with the *Streptococcus* in one or two cases show a slight parallelism with those obtained in animal experiments, but with the majority of compounds the antiseptic value shows little correlation with the curative power.

Apart from causing a slight delay in the growth rate in a few experiments, the compounds give almost uniformly negative results with the *Staphylococcus* and *Bacillus aertrycke*.

DISCUSSION AND CONCLUSIONS

Perhaps the most interesting result of the work here reported is the relatively high antistreptococcal activity of 3-nitro-4-hydroxybenzenesulphonamide. All the sulphur-containing compounds previously found to exercise marked chemotherapeutic activity against septicaemias caused by the haemolytic *Streptococcus* have an amino group or a group which can be easily converted into the amino group, e.g. the nitro group, either free or substituted in the *p*-position in the benzene ring. The activity of the new compound indicates that this rule is not general and suggests that it is important to prepare and test other derivatives possessing the 3-nitro-4-hydroxybenzene grouping. It will be noted that 3-nitro-4-hydroxybenzenesulphonamide is definitely less toxic than sulphanilamide. This compound was also active on pneumococcal septicaemias in mice but in this case its efficacy was definitely inferior to that of *p*-aminobenzenesulphonamide.

p-Hydroxybenzenesulphonamide, as well as certain disubstituted benzenesulphonamides, have been tested by Tréfouel *et al.* [1937] in respect of their antistreptococcal activity in mice, in every case with practically negative results. Our results with the first of these compounds are in agreement with their findings; of the disubstituted derivatives used by Tréfouel *et al.* none were available to us.

The antistaphylococcal activity of 4:4'-diacetyldiaminodiphenylsulphide is of some interest. Though the activity of this compound against the *Staphylococcus* is not very great, under favourable conditions it does delay death by about 4 days, and this indicates that antistaphylococcal action is not limited to certain sulphonamide derivatives but belongs also to other classes of sulphur-containing compounds.

The main conclusion to be drawn from a comparison of the experiments carried out on infected mice and those performed *in vitro* is a negative one. There is no obvious connexion, as far as our results show, between antibacterial actions under these two different conditions. Thus it would appear that the effect of the compound on the bacteria which is of importance for its chemotherapeutic action is not one of general toxicity, i.e. a simple inhibition of growth or metabolism, but is rather a highly specific and characteristic one. The view that this group of active sulphur-containing compounds exercise their chemotherapeutic action through some special damaging effect on the resistance of the organisms to the antibacterial action of the blood and the body tissues has been advocated by many investigators, and has recently received support from the observations of Whitby [1938]. He has shown that the remarkable chemotherapeutic action of 2-*p*-aminobenzenesulphonamidopyridine on mice infected with pneumococci is associated with the loss by the bacteria of their capsule which makes the organisms much more susceptible to phagocytosis and other similar processes. The balance of the evidence seems to suggest that some similar though perhaps

more subtle change is effected in organisms such as the *Streptococcus* by compounds of the sulphonamide group and other active sulphur-containing derivatives.

SUMMARY

1. Ten compounds have been tested in respect of their chemotherapeutic actions on mice infected with haemolytic streptococci, pneumococci, staphylococci or *Bacillus aertrycke*. The antibacterial actions *in vitro* of these compounds have also been examined.

2. 3-Nitro-4-hydroxybenzenesulphonamide has a marked curative effect on streptococcal septicaemias in mice. The low toxicity of this compound suggests that it should be investigated on a wider scale.

3. Diacetyldiaminodiphenylsulphide delays the death of mice infected with staphylococci.

4. Under the conditions of experimentation, no correlation could be established between the action of these compounds *in vitro* and their chemotherapeutic activity on infected mice. This result is in accordance with the view that the important action of these compounds on the organisms is not one of gross toxicity, but that it involves a damaging of the defences which normally protect the organisms from the bactericidal processes of the body.

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CCXXXI. THE COMPONENT GLYCERIDES OF AN OX DEPOT FAT

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THE general composition of the depot fats of the ox and the pig was indicated by Banks & Hilditch [1931; 1932] by consideration of the proportion of fully saturated glycerides present in these fats together with the percentage composition of the fatty acids in the whole fats and in their fully saturated glycerides. Later, in the case of a pig depot fat, Hilditch & Stainsby [1935] obtained somewhat more detailed information by its study after it had been progressively hydrogenated to varying extents, coupled with the determination of tristearin in the completely hydrogenated fat. More recently it has been found possible to obtain an approximate estimate of at all events the main component glycerides present, in solid fats of comparatively simple fatty acid composition, by preliminary systematic crystallization of the fat from acetone. This procedure, although usually incapable of yielding definite individual mixed glycerides, causes a division of the fat into sparingly soluble portions in which mono-unsaturated-disaturated (and fully saturated) glycerides predominate, and more soluble portions in which the di-unsaturated glycerides (and tri-unsaturated glycerides when present) are concentrated. The fat is thus divided into two or three fractions, each of which is investigated as follows:

(*a*) the component acids are determined by ester-fractionation;

(*b*) a portion is hydrogenated and the tristearin content of the product determined;

(*c*) where necessary, the fully saturated glycerides present are isolated and determined: the component acids present in the fully saturated glycerides, and the tristearin content of the latter, are determined.

In addition to the data for fully saturated glycerides, it is then possible, in each fraction of the fat, (i) to estimate the proportions of mono- and of di-unsaturated glycerides (or of di- and tri-unsaturated glycerides) and (ii), knowing the tri-C_{18} glyceride content (determined as tristearin in (*b*)), to estimate the proportions of mono-C_{18}- and di-C_{18}-mixed glycerides in which another homologous acid (palmitic) is present. With these data, and taking into account the known general order of solubility in acetone of, for example, oleodistearin, dioleostearin, oleopalmitostearin, oleodipalmitin and palmitodiolein, it is usually possible to give a detailed, approximately quantitative statement of the component glycerides in each portion of the fat, and therefrom to deduce that of the whole fat.

This method has been used in the investigation of a number of solid seed fats, including cacao butter, mowrah fat and shea fat, by Hilditch *et al.* [1936; 1938, 1, 2, 3]. The present communication describes its application to a typical ox depot fat. Here the procedure is somewhat more complicated owing to the comparatively large proportions of fully saturated glycerides present, and to the circumstance that animal depot fats contain a somewhat greater number of minor component acids than any of the seed fats to which the method has yet been applied.

The ox depot fat employed was obtained by extracting with acetone the perinephric tissue from a Shorthorn heifer. It had i.v. (Wijs) 38·7, sap. equiv. 284·0, and contained 0·12 % of unsaponifiable matter. The fatty acids (185·9 g.) obtained on hydrolysis of 200 g. of the fat were submitted to lead salt separation by the modified Twitchell method [Banks *et al.* 1933], and the resulting acid fractions were converted into methyl esters and fractionally distilled. The esters of the "solid" acids were distilled from a Willstätter bulb; those of the "liquid" acids were distilled through the electrically heated and packed column described by Longenecker [1937]. The final results of the analysis are summarized in Table I.

Table I. *Component acids of ox depot fat*

Component acids	"Solid" (56·6 %)	"Liquid" (43·4 %)	Total	% (wt.)	% (mol.)
Saturated:					
Lauric	—	0·50	0·50	0·5	0·7
Myristic	0·68	2·04	2·72	2·7	3·2
Palmitic	27·17	3·23	30·40	30·4	32·2
Stearic	23·63	--	23·63	23·7	22·6
Unsaturated:					
Tetradecenoic	—	0·41	0·41	0·4	0·5
Hexadecenoic	---	1·65	1·65	1·7	1·8
Oleic	5·08	33·49	38·57	38·6	37·1
Octadecadienoic	—	2·00	2·00	2·0	1·9
Unsaponifiable:	0·04	0·08	0·12	--	---

Crystallization of the ox depot fat from acetone

About 1 kg. of the fat was subjected to systematic crystallization from acetone at 0° or room temperature as follows.

(i) The fat (in three portions for convenience) was dissolved in acetone (5 ml. per g. of fat) and kept at room temperature overnight; after filtering the separated fat the solution was then cooled at 0° overnight and a further fraction of fat separated:

Fat taken g.	Separated at room temperature			Separated at 0			Left in solution at 0°		
	No.	g.	I.V.	No.	g.	I.V.	No.	g.	I.V.
350	A_1	102	22·8	B_1	130	35·7	C_1	118	56·3
350	A_2	95	16·9	B_2	161	42·2	C_2	94	57·6
325	A_3	96	19·3	B_3	142	41·6	C_3	87	57·6

(ii) Corresponding fractions were then united and further crystallized as shown in the scheme below:

Fractions crystallized		Conditions of crystallization			Separated fat			Fat left in solution		
Nos.	g.	Acetone (ml. per g. fat)	Temp.	Time (hr.)	No.	g.	I.V.	No.	g.	I.V.
$A_1 + A_2 + A_3$	293	3 : 1	Atmospheric	16	A_4	244	15·9	A_5	49	41·1
$B_1 + B_2 + B_3$	433	5 : 1	0°	6	B_4	347	37·1	(further 16 hr. at 0°)		
			0°	16	B_5	27	40·4	B_6	59	55·3
$A_5 + B_5$	76	5 : 1	0°	6	D_1	66	40·6	D_2	10	53·8

The eight fractions (A_4, B_4, B_6, D_1, D_2, C_1, C_2, C_3) finally obtained were then assembled into three groups of similar I.V. as follows:

No.	g.	I.V.	No.	g.	I.V.
A	244	15·9	A_4	244	15·9
B	413	37·5	B_4	347	37·1
			D_1	66	40·6
C	368	57·4	C_1	118	56·3
			C_2	94	57·6
			C_3	87	57·6
			B_6	59	55·3
			D_2	10	52·8

(The weights actually obtained were slightly diminished owing to withdrawal of samples for I.V. determinations, etc., and have been "corrected" in the above description to allow for this; the total weight so withdrawn amounted to about 5 g., or 0·5% of the original fat employed.)

The component fatty acids in each of the fractions A, B, C were determined by lead salt separation and ester-fractionation as in the case of the whole fat (Table I). The final percentage compositions (wt. and mol.) found for the acids from each fraction are shown in Table II.

Table II. *Component acids of the separated fractions (A, B, C) of ox depot fat*

	A		B		C	
	% (wt.)	% (mol.)	% (wt.)	% (mol.)	% (wt.)	% (mol.)
Saturated:						
Lauric	--	--	0·2	0·2	0·3	0·4
Myristic	1·3	1·5	2·1	2·6	2·5	3·0
Palmitic	44·0	46·5	29·8	31·7	25·0	26·5
Stearic	31·8	30·3	25·8	24·7	12·2	11·7
As arachidic	5·5	4·8	0·4	0·3	0·1	0·1
Unsaturated:						
Tetradecenoic	0·3	0·4	0·3	0·4	0·7	0·9
Hexadecenoic	1·1	1·1	1·5	1·6	2·6	2·7
Oleic	16·0	15·4	38·8	37·4	47·4	45·8
Octadecadienoic	-	-	1·1	1·1	9·0	8·8
As C_{20-22} unsaturated	-	-	—	—	0·2	0·1

It will be observed that, of the minor component acids, arachidic acid is the only one concentrated in fraction A, in which fully saturated and mono-unsaturated-disaturated glycerides predominate. Myristic, tetra- and hexa-decenoic, and especially octadecadienoic and the unsaturated C_{20-22} acids, are found in the (mainly di-unsaturated) glycerides of fraction C, which are liquid at room temperature and most soluble in acetone.

Examination of fully saturated glycerides present in fractions A and B of the ox depot fat

The fully saturated glycerides were isolated, and their amounts determined, in each case by oxidation of the fats in acetone solution with powdered $KMnO_4$ by our usual procedure.

Fraction A: 80·2 g. gave 49·9 g. fully saturated glycerides (sap. equiv. 283·4, acid value 4·8, I.V. 0·2). *Fully saturated glycerides*: 59·0% (wt.) or 60·0% (mol.).

Fraction B: 200 g. gave 14·6 g. fully saturated glycerides (sap. equiv. 272·1, acid value 1·8, I.V. 0·4). *Fully saturated glycerides*: 7·1% (wt.) or 7·4% (mol.).

The component acids in each fraction of fully saturated glycerides were determined by ester-fractionation with the results given in Table III.

Table III. *Component acids of the fully saturated glycerides in fractions*
A and B

	A		B	
	% (wt.)	% (mol.)	% (wt.)	% (mol.)
Myristic	5·4	6·3	7·5	8·6
Palmitic	49·6	51·5	63·0	64·2
Stearic	45·0	42·2	29·5	27·2

A portion of the fully saturated glycerides from the least soluble fraction A of the ox fat was systematically crystallized from anhydrous ether into five fractions. From the sap. equiv. of these fractions it was estimated that the material contained 3·0% (mol.) of tristearin and 19·6% (mol.) of tripalmitin (the latter figure is probably somewhat higher than the truth, because traces of acidic products of oxidation left in the fully saturated glycerides may have lowered the equiv. of the portion most soluble in ether).

Lack of material prevented similar crystallization of the fully saturated glycerides from fraction B, which however only amounted to 3% of the whole fat, and were taken as a mixture of tripalmitin and dipalmitostearin in the proportions demanded by the fatty acid composition.

Determination of tri-C_{18} glycerides in each fraction (A, B, C) of the ox depot fat

A portion (50 g.) of each fraction was completely hydrogenated and then submitted to systematic crystallization from anhydrous ether. From the sap. equiv. of the six or seven crystal fractions thus obtained the proportion of tristearin was determined [cf. Hilditch & Stainsby, 1935]. The molar percentage, of course, corresponds with the molar percentage of glycerides composed exclusively of C_{18} acids in the original fats before hydrogenation. The amounts of tristearin determined by this procedure in the hydrogenated portions A, B and C are shown in Table IV.

Table IV. *Tristearin content of hydrogenated fractions A, B and C of the*
ox depot fat

Fraction	% (wt.)	% (mol.)
A	18·4	17·7
B	15·6	15·1
C	9·7	9·3

DISCUSSION

The evaluation of the data recorded in the experimental part requires some explanation both as regards general interpretation and as regards the manner in which the various minor component acids in the fat are considered. The discussion is based throughout, it should first of all be noted, on the molar (not weight) proportions of the various components: this has the primary advantage that in any given case the same numerical figure denotes the proportion of a component either in the form of glyceride or of the fatty acid in question. The data for the molar proportions of glycerides in fractions A, B and C of the ox depot fat, and for the increments of component acids present in each fraction (from the percentage figures given in Table II), are shown in Table V.

Comparison of the last column in Table V with Table I shows satisfactory accordance in the percentages of the component acids as determined (i) in the whole fat and (ii) from summation of the component acids recorded for each of the three fractions A, B and C. As would be expected, the components present in very small proportions are more apparent in the data for the subdivided parts,

Table V. *Proportions (mol. %) of glycerides and of their component acids in fractions A, B and C*

	A	B	C	
Weight of fraction:	244	413	368	
I.V.	15·9	37·5	57·4	
Sap. equiv.	283·3	284·9	285·3	
Unsaponifiable %	——	0·06	0·2	
Glycerides % (wt.)	23·8	40·3	35·9	
Glycerides % (mol.)	23·9	40·3	35·8	
Component acids (increments % mol):				Whole fat
Lauric	—	0·1	0·15	0·25
Myristic	0·35	1·0	1·05	2·4
Palmitic	11·1	12·8	9·5	33·4
Stearic	7·2	10·0	4·2	21·4
As arachidic	1·15	0·1	0·05	1·3
Tetradecenoic	0·1	0·2	0·3	0·6
Hexadecenoic	0·3	0·6	1·0	1·9
Oleic	3·7	15·1	16·4	35·2
Octadecadienoic	- -	0·4	3·1	3·5
$C_{20\ 22}$ unsaturated	——	- -	0·05	0·05

in one or other of which they have become concentrated. Indeed, arachidic and unsaturated acids of the C_{20} and C_{22} series (which are found only in the residual fractions of the distilled esters) only became detectable in the analyses of the fat after separation by crystallization.

The method of estimation of component glycerides employed in this work depends essentially on comparison of non-C_{18} and C_{18} acid components on the one hand, and of the total saturated and unsaturated acid components on the other. Complete detailed results can therefore only be obtained when the acids of the fat consist of one saturated non-C_{18} acid (e.g. palmitic), one saturated C_{18} acid (stearic) and unsaturated C_{18} acids (which, whether oleic or linoleic, etc., must be considered for the present purpose as one group; it is clearly impossible, by the use of methods involving hydrogenation, to distinguish between what were originally oleic or linoleic glycerides. To emphasize this, as in previous papers, the prefixes "oleic" or "oleo-" are employed when it is desired to indicate that all the C_{18} unsaturated components, and not merely oleic derivatives alone, are included).

In the seed fats investigated by this procedure, the acids present have been confined so far to palmitic, stearic, oleic and linoleic, with the exception of extremely small proportions of myristic or arachidic acids. In the case of ox depot fat (and of other animal fats which we are at present examining) the number of these minor components is increased and unfortunately includes, amongst others, about 2% of hexadecenoic acid (and traces of tetradecenoic acid), belonging simultaneously to the unsaturated group and to the group of non-C_{18} acids. In relation to the three major components (palmitic 33, stearic 21, oleic 35 ("oleic" 39), amounting together to 93% of the component acids) the quantity of any one of the minor components is almost insignificant; but it must be remembered that each mol. of a minor component will be associated almost invariably with 2 mol. of one or other of the three major components in a mixed triglyceride molecule, so that the total percentage of triglycerides involved is not of the order of 7%, but about 20%, of the whole fat. Whilst it is desirable to draw attention to this feature, it should equally be pointed out that the data presented in this paper are entirely valid from the point of view of the distribution of saturated non-C_{18} and C_{18} acids in the mixed glycerides of the depot fat,

and that the great predominance of palmitic acid in the former class ensures that the conclusions drawn later cannot be, in point of fact, very far from the actual state of affairs. It is perhaps not unreasonable to remark upon the fact that the presence of a number of minor component acids is the bugbear of detailed study of component glycerides in natural fats; in the rare absence of these, it is now possible to give an almost exact statement of the component glycerides present in a fat containing only three fatty acids.

For the purpose of the present discussion, myristic and lauric are included with palmitic glycerides, and arachidic with stearic glycerides, whilst the traces of unsaturated C_{20} and C_{22} acids are included in the "oleic" group. Hexadecenoic (and tetradecenoic) glycerides, which fall in the unsaturated non-C_{18} acid category, have been included also with palmitic glycerides, since on hydrogenation (for the tri-C_{18} glyceride determinations) they yield palmito- (or myristo-) glycerides. Accordingly, in terms of non-C_{18} ("palmitic"), stearic and "oleic" derivatives, the data in Table V, together with those for the fully saturated glycerides and contents of tri-C_{18} glycerides, may be transformed as shown in Table VI. In Table VI are also given the deduced proportions of palmitodi-C_{18}- and dipalmitomono-C_{18}-glycerides, and of mono- and di-unsaturated glycerides. Since the determination of the former and of tristearin, by crystallization of the hydrogenated fats from ether, is of a lower order of accuracy than the component acid analyses in Table II, the palmitodi-C_{18}- and dipalmitomono-C_{18}-glycerides have been calculated from the latter, after deduction of the proportion of tri-C_{18} acids present as tri-C_{18} glycerides. In fractions A and B, the fully saturated glycerides present are of course first dealt with, and the remaining acids accounted for in the various categories of mixed saturated-unsaturated glycerides.

Table VI. *Glyceride categories (°/₀ mol.) present in fractions A, B and C of ox depot fat*

	A		B		C	Whole fat
Glycerides	23·9		40·3		35·8	100·0
Component acids (increments):						
Palmitic	11·85		14·7		12·0	38·55
Stearic	8·35		10·1		4·25	22·7
"Oleic"	3·7		15·5		19·55	38·75
Component glycerides (increments):	Fully saturated	Mixed	Fully saturated	Mixed		
Tri-C_{18}	0·4	3·8	—	6·1	3·3	13·6
Palmitodi-C_{18}	5·8	0·8	—	24·9	29·0	60·5
Dipalmitomono-C_{18}	5·4	4·9	2·4	6·3	3·5	22·5
Tripalmitin	2·8	--	0·6	—	—	3·4
Mono-"oleo"-disaturated	—	8·0	—	28·1	12·9	49·0
Di-"oleo"-monosaturated	—	1·5	—	9·2	22·9	33·6

Consideration of the data in Table VI leads us to the statement of component glycerides in the fractions of the original fat, and consequently in the whole of the original fat, given in Table VII. These considerations are as follows.

Fraction A. The fully saturated components are derived from the estimated amounts of tripalmitin and tristearin, those of dipalmitostearin and palmito-distearin following from the component acid percentages in Table III. In the mixed saturated-unsaturated glycerides, the 4·9 % of dipalmitomono-C_{18} glycerides must be "oleo"-dipalmitin. Of di-"oleo"-monosaturated glycerides, palmitodi-"oleins" are relatively soluble in acetone and may safely be considered absent from the sparingly soluble fraction A; the 1·5 % of di-"oleo"-.

glycerides is therefore credited as stearodi-"olein" and the remaining 2·3 % of tri-C_{18} glycerides as "oleo"-distearin, whilst the 0·8 % of palmitodi-C_{18} glycerides is taken as "oleo"-palmitostearin.

Fraction B. After reckoning the small amount of fully saturated glycerides (from the fatty acid analysis, Table III) as tripalmitin (0·6) and dipalmitostearin (2·4), and the remaining 6·3 % of dipalmitomono-C_{18} glycerides as "oleo"-dipalmitin, the 6·1 % of tri-C_{18} glycerides is assumed to be stearodi-"olein". (Some of the latter might be, perhaps, "oleo"-distearin, but since the amount of this relatively insoluble glyceride in the least soluble fraction A is only 2·3 % of the whole fat, it is certain that the proportion in fraction B will be definitely less than this.) Of the 9·2 % of di-"oleo"-glycerides, the remaining 3·1 % is then palmitodi-"olein", and the rest of the 28·1 % of mono-"oleo"-glycerides appears as "oleo"-palmitostearin.

Fraction C. From the proportions of saturated and unsaturated acids in this portion, its components lie between the following limits:

	% (of whole fat)
Mono-"oleo"-glycerides	12·9–24·4
Di-"oleo"-glycerides	22·9– 0
Tri-"oleins"	0–11·4

Since, however, the total amount of tri-C_{18} glycerides in C is only 3·3 % of the whole fat, there cannot at most be more than 3·3 % of tri-"olein" present (the corresponding figures in this case being 16·3 % of palmitodi-"olein", with 12·7 % of "oleo"-palmitostearin). But the presence of probably nearly 6 % of stearodi-"olein" in fraction B implies almost certainly that appreciable amounts of this glyceride will be present in the most soluble fraction C, and therefore, whilst the experimental data do not permit a final subdivision into tri-"olein" and stearodi-"olein", the probability is very great that nearly all the 3·3 % of tri-C_{18} glycerides in fraction C is, in fact, stearodi-"olein". The experimental figures, in fact, show almost conclusively that tri-"oleins", if not wholly absent, can only be present in quite insignificant amounts in the specimen of ox depot fat investigated.

The proportions of the remaining components of fraction C, once the nature of the tri-C_{18} unsaturated glycerides has been settled, follow as in the preceding cases of fractions A and B.

Table VII. *Probable component glycerides (% mol.) of the ox depot fat*

	A		B		C	Whole fat	
	Fully saturated	Mixed	Fully saturated	Mixed		Exact figure	(in round nos.)
Fully saturated glycerides (17·4 %):							
Tripalmitin	2·8	—	0·6	—	—	3·4	3
Dipalmitostearin	5·4	—	2·4	—	—	7·8	8
Palmitodistearin	5·8	—	—	—	—	5·8	6
Tristearin	0·4	—	—	—	—	0·4	†
Mono-"oleo"-disaturated glycerides (49·0 %):							
"Oleo"-dipalmitin	—	4·0	—	6·3	3·5	14·7	15
"Oleo"-palmitostearin	—	0·8	—	21·8	9·4	32·0	32
"Oleo"-distearin	—	2·3	—	—	—	2·3	2
Di-"oleo"-monosaturated glycerides (33·6 %):							
Palmitodi-"oleins"	—	—	—	3·1	19·6	22·7	23
Stearodi-"oleins"	—	1·5	—	6·1	3·3	10·9	11
Tri-"oleins"	—	—	—	—	*	*	*

* Traces of triolein, probably not exceeding 1 % of the fat, may be present.
† Tristearin is present to the extent of less than 1 % of the fat.

The ox depot fat investigated thus consists of about one-third of "oleo"-palmitostearins, and one-quarter of palmitodi-"oleins", with (in progressively diminishing proportions) "oleo"-dipalmitin, stearodi-"olein", dipalmitostearin and palmitodistearin. Very small amounts of tripalmitin, tristearin and (possibly) tri-"olein" are also present.

It is interesting to compare the detailed results obtainable by the procedure which has now been employed with those obtained by earlier methods. By simple but intensive crystallization of a sheep depot fat, Bömer et al. [1907; 1909] detected about 3% of tristearin with 4–5% of dipalmitostearin and some palmitodistearin. Banks & Hilditch [1931; 1932] were able to give limiting values for the mixed saturated-unsaturated glycerides by determining the total molar proportions of the fully saturated glycerides present. In the ox depot fat which has now been studied this procedure would have enabled us only to state the approximate components as fully-saturated 17·4%, mono-"oleo"-disaturated from 49 to 66%, di-"oleo"-monosaturated from 34 to 0% and tri-"oleins" from 0 to 17%. Determination of the tri-C_{18} glyceride content of the whole fat, coupled with consideration of the major component acids and the fully saturated glycerides, as applied by Hilditch & Stainsby [1935] to a pig depot fat, would have permitted a somewhat narrower range of the chief possible components: for example, 0–13·6% tri-"olein", 13·6–0% stearodi-"olein", 20–7% palmitodi-"olein", 34–47% "oleo"-palmitostearin, with 15% "oleo"-dipalmitin and 17·4% of fully saturated glycerides.

The procedure which has now been described, however, has demonstrated that tri-"oleins" are unlikely to be present in more than traces, and cannot in any case form more than 3% of the fat: whilst it has also indicated the presence of very small proportions (about 2%) of "oleo"-distearin. It therefore follows that the proportions of each of the component glycerides are defined by the approximate figures given in the final column of Table VII. (Even if the 3% of tri-C_{18} glycerides in fraction C were calculated alternatively as stearodi-"oleins" and as tri-"oleins", the possible limits of the component glycerides concerned would be narrowed to 0–3% tri-"olein", 11–8% stearodi-"olein", 23–20% palmitodi-"olein" and 32–35% "oleo"-palmitostearin.)

The possession of approximate figures for the chief component glyceride percentages permits some further discussion of the characteristic glyceride structure of ox depot fat in contrast to the "evenly distributed" type of glyceride structure encountered in the majority of natural fats. As mentioned earlier, the glycerides of several solid seed fats which conform closely to the "evenly-distributed" type have been investigated by the present method, and it has been found [Hilditch, 1938] that the observed proportions therein of the di-"oleo"-glycerides, and to a less extent the mono-"oleo"-glycerides, can be fairly closely reproduced by a simple calculation based upon the proportions of the component acids in the whole fat. If the chief component acids of the ox depot fat were assembled into a mixture of component glycerides on the lines of a typical seed fat (in which case there would be negligible proportions of fully saturated glycerides present), the application of the calculation referred to would suggest the probable presence of about 10% palmitodi-"olein", 6% stearodi-"olein", up to 62% of "oleo"-palmitostearin and 22% or more of "oleo"-dipalmitin—proportions which, naturally, differ widely from those observed in the foregoing analysis. If, on the other hand, allowance is made for the 17·4% of fully saturated glycerides known to be present, application of the numerical calculation to the component acids (28·0% palmitic, 15·8% stearic and 38·8% "oleic") of the mixed saturated-unsaturated glycerides would suggest the following composition

for the latter: 22 % palmitodi-"olein", 12 % stearodi-"olein", up to 35 % of "oleo"-palmitostearin and 14 % or more of "oleo"-dipalmitin. These figures are remarkably close to those actually observed (Table VII), but the implication of the coincidence, if it be more than a fortuitous one, is not at present altogether clear. Until the results of some contemplated parallel investigations of other animal depot and milk fats become available, it is therefore deemed desirable merely to draw attention to this feature without further comment, other than that an "even distribution" of the component acids of the mixed saturated-unsaturated glycerides seems not inconsistent with the hypothesis [Hilditch & Sleightholme, 1931; Banks & Hilditch, 1932; Hilditch, 1937, 1, 2] that the stearo-glycerides in the stearic-rich animal depot fats have resulted from saturation of preformed oleo-glycerides.

The hypothesis in question (with its extension to milk-fat glycerides) correlates the unusually large proportions of fully saturated glycerides present in many animal depot and milk fats with their possible formation by hydrogenation (depot fats) or other transformation (the lower saturated acids of milk-fats) of oleic groups already in combination as glycerides. The results of the present study may be briefly discussed in relation to this view. The ox depot fatty acids include, in molar proportions (Table VI), 38·5% palmitic (or, more strictly, non-C_{18} saturated acids), 22·7 % stearic and 38·8 % oleic (with octadeca-dienoic) acid, and of the glycerides 13·6 % contain three C_{18}, 60·5 % contain two C_{18}, and 22·5 % contain only one C_{18} acyl group. The observed composition (Table VII) of these various groups was as follows:

	Tri-"oleo"	Di-"oleo"	Mono-"oleo"	Fully saturated
Tri-C_{18} glycerides	Nil or traces	10·9	2·3	0·4
Palmitodi-C_{18} glycerides	—	22·7	32·0	5·8
Dipalmitomono-C_{18} glycerides	—	—	14·7	7·8

If, in accordance with the above hypothesis, it be assumed that all these products have resulted from hydrogenation of preformed oleo-glycerides, their percentage proportions, at corresponding stages of the supposed hydrogenation, would be as below:

From	Unchanged %	1st stage %	2nd stage %	3rd stage %
Tri-"oleins"	Nil or traces	80	17	3
Palmitodi-"oleins"	37	53	10	—
Dipalmito-"oleins"	65	35	—	—

In catalytic hydrogenation of mixtures of mono-, di- and tri-oleo-glycerides in presence of nickel at 170–180° it has been shown [Hilditch & Jones, 1932; Bushell & Hilditch, 1937] that the process takes place in consecutive stages (i.e. only one oleo-group is saturated at a time) and that, whilst all three stages of hydrogenation proceed concurrently, the reduction of tri-oleo- to di-oleo-glycerides proceeds much more rapidly than that of di-oleo- to mono-oleo-glycerides, and the latter probably proceeds somewhat more rapidly than the production of saturated from mono-oleo-glycerides. Further, the relative concentrations of the different groups have an important influence on the amounts of the various semi-hydrogenated glycerides produced. The observed proportions of these products (illustrated in percentage form above) is that which would be expected if a biological hydrogenation process had followed the same course as addition of hydrogen by a nickel catalyst. Little or no tri-unsaturated glycerides are present, and the proportions of glycerides corresponding with the second and third stages of hydrogenation are much less than those of the initial

stages. Less of the original oleodipalmitin (as presupposed by the hypothesis) has been reduced than of the original palmitodiolein, but the amount of the latter present is nearly three times that of the former, and this is therefore also in keeping with the above-mentioned observations.

The observed presence of perhaps 2–3 % of tripalmitin in a fat which contains large proportions of stearic as well as palmitic acid is unusual. Tripalmitin has hitherto only been reported in cases in which the saturated acids of a natural fat consist almost wholly of palmitic acid (e.g. olive oil, palm oil, rabbit depot fat), and, when stearic and palmitic acids are both present in quantity (as in cacao butter and many other seed fats), any fully saturated components have been found to consist of mixed palmitostearins. The tripalmitin in ox depot fat is explicable, however, on the above "hydrogenation" hypothesis, since hexadecenoic acid is a minor component of ox depot fat, and saturation of any hexadeceno-palmitins present in the fat would, of course, yield tripalmitin.

SUMMARY

A specimen of ox depot fat has been separated into fractions of varying degrees of solubility in acetone, and the component acids and glycerides present in each fraction have been investigated. The procedure leads to an approximate statement of the proportions of each of the major component mixed glycerides present in the fat. The approximate molar percentages of the more abundant glycerides are "oleo"-palmitostearin 32, palmitodi-"olein" 23, "oleo"-dipalmitin 15 and stearodi-"olein" 11 %; 17 % of fully-saturated glycerides are present, mainly dipalmitostearin and palmitodistearin, with very small amounts of tripalmitin and tristearin. Triolein is either absent, or only present in very small quantities. (Myristic, hexadecenoic, arachidic and other acids which are present to very small extents in the fat have to be included with palmitic acid in the analysis of the component glycerides.)

The proportions of the respective groups of tri-, di- and mono-"oleo"-glycerides, and of the fully-saturated glycerides, are fully consistent with the hypothesis that the stearo-glycerides in ox depot fat have resulted from saturation of preformed oleo-glycerides.

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CCXXXII. STANDARDIZATION OF THE RAT FOR BIO-ASSAY OF VITAMIN E[1]

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A SURVEY of numerous studies concerned with the experimental production of vitamin E deficiency reveals a rather wide variation in the time necessary to induce sterility in rats of either sex. In male rats reared from weaning on vitamin E-deficient diets, the time of experimental feeding necessary to produce degenerative changes in the germinal epithelium may vary from 50 days or less [Mason, 1926; Ringsted, 1936] to 90–150 days [Evans & Burr, 1927; Kudrjaschov, 1930; Juhász-Schäffer, 1931]. It has also been the experience of many investigators that a variable proportion of female rats reared on vitamin E-deficient diets will complete one or more pregnancies before exhibiting a resorption-gestation typical of vitamin E depletion. This variable occurrence of initial fertility has proved particularly annoying and time-consuming in the preparation of rats for the bio-assay of vitamin E, and has necessitated the production of a proven resorption in all rats prior to their use in assay tests. Attempts to eliminate the occurrence of this phenomenon by the use of slightly rancid fat in the hopes of destroying possible traces of vitamin E in the experimental diets used have not proved particularly effective. Furthermore, such a procedure seems highly undesirable in view of the observations [Kudrjaschov, 1935] that products of a ketone nature formed in rancid fats are capable of preventing implantation in rats and may even cause resorption of the foetus if implantation is successful. Proper limitation of the vitamin E content of the diet supplied to mothers whose offspring are to be selected for vitamin E deficiency studies appears to be a much more successful and acceptable method for elimination of initial fertility [Ringsted, 1936], but its general applicability is limited by the care and expense involved in the preparation of stock diets containing a uniformly low content of vitamin E. More recently, Bacharach *et al.* [1937] report the occurrence of resorption-gestations in the first pregnancies of 96 out of 104 vitamin E-deficient rats examined prior to 1937, and in 100 % of 316 rats tested since that time, but fail to mention the extent to which the lactating young had access to the maternal diet or the composition of the latter.

Although it has been rather generally accepted that the vitamin E reserves of young rats and the variable response of the latter to vitamin E depletion are significantly influenced by the vitamin E content of the maternal diet, no effort has been made to evaluate the mechanisms concerned. The present communication deals with an attempt to determine to what extent the vitamin E reserves of newly weaned rats are due to direct consumption of the maternal diet as the young adopt free-eating habits during the latter part of lactation, and to what

extent they can be attributed to placental and mammary transfer. These studies have also demonstrated the reliability of a relatively simple procedure which completely eliminates the occurrence of initial fertility and greatly facilitates the preparation and standardization of rats to be used in the bio-assay of vitamin E, or for experimental studies relating to vitamin E deficiency.

Vitamin E storage in offspring given access to maternal diet

In order to determine the storage capacity for vitamin E in suckling rats, the offspring of mothers maintained on diets containing variable amounts of vitamin E were permitted access to the maternal diet during the period of lactation, after which they were reared upon a vitamin E-deficient diet (diet 68, Table II). The data summarized in Table I demonstrate conclusively that delayed

Table I. *Onset of sterility in vitamin E-deficient rats in relation to the vitamin E content of the diet available during lactation*

Group	No. of rats	Diet to which rats had access before vitamin E depletion was begun	Age when put on vitamin E-deficient diet days	Days on vitamin E-deficient diet before onset of sterility† Min.	Max.	Average
A	24	Stock diet,* supplemented with a	21–30	160	324	240
B	10	special suckling diet containing 20% ether-extracted wheat germ still containing appreciable amounts of vitamin E	25–29	150	200	180
C	13	Stock diet, supplemented with	21	90	218	151‡
D	18	fresh lettuce 2–3 times weekly	21	95	160	130
E	10	Stock diet only	21	58	113	82§
F	5		21	80	100	90
G	178	Vitamin E content of maternal	21	32	71	43·4‖
H	118	diet unrestricted and often excessive, but mother and offspring given vitamin E-deficient diet after 10th day of lactation	21	30	70	48·4

* The stock diet used in these studies was a commercial dog biscuit which affords a daily intake of from 2–3 times the minimal requirement of vitamin E for the rat.

† Onset of sterility in female rats was taken as the beginning of the first gestation period during which resorption occurred. In males this was estimated on the basis of histological examination of the testis and epididymis and can be regarded as only approximate.

‡ Two rats resorbed during first gestation, 5 completed one, 3 completed two and 3 completed three gestations before resorption occurred.

§ Resorption during first gestation occurred in 8 out of 10 rats.

‖ First litter fertility completely eliminated.

onset of sterility in rats of both sexes is roughly proportional to the vitamin E content of the maternal diet, when the young have access to the latter throughout the period of lactation. The particularly long depletion period in the rats of groups A and B is due partly to the higher content of vitamin E in the diet, as compared with the other groups, and partly to maintenance on the maternal diet beyond the 21st day of life (at which time the rats in our colony are as a matter of routine weighed, removed from the mother and placed upon the experimental diets). Attempts to hasten the onset of sterility in a number of animals in groups A to D by aeration of the lard at 100° for 10 hr. before its inclusion in the diet were unsuccessful. Even the unsupplemented stock diet conferred an appreciable storage in suckling rats permitted to consume it prior to the 21st day of life (groups E and F). However, when the offspring were denied access to the maternal diet and consumed only a vitamin E-deficient diet from the

assumption of free-eating habits, sterility supervened very early (groups G and H). The latter two groups represent a summary of results obtained in the experiments discussed in the following sections.

Vitamin E storage in offspring denied access to the maternal diet

Three groups of 13 rats each, containing an equal distribution of litter-mate sisters, were selected for breeding and reared upon the diets S, S + E and D + E, respectively (Table II). It was estimated that the S diet supplied approximately 2-3 times the minimal requirement of vitamin E for the rat, as determined by bio-assay of known quantities of the diet and measurements of the daily food consumption. It was estimated that the S + E diet supplied approximately

Table II. *Composition of diets*

Diet S		Diet S + E	
Stock diet (commercial dog biscuit)		Stock diet	80
		Fresh wheat germ	20
Diet D + E		Diet 68	
Casein*	22	Casein*	22
Cornstarch	54	Cornstarch	54
Lard	20	Lard	20
Salts†	4	Salts	4
Brewer's yeast	1 g. daily	To each 100 g. of this diet, 8 g. brewer's yeast and 2 g. cod liver oil were added at the time of feeding	
Cod liver oil	6 drops daily		
Wheat germ oil	6 ,,		

* Finely granulated commercial casein found to be suitable for use in vitamin E deficient diets without further purification.

† Modification of the Osborne-Mendel salt mixture suggested by Wesson [1932].

‡ Kindly supplied by General Mills, Inc., Research Laboratories, Minneapolis, Minn.

15 times, and the D + E diet about 10 times, the minimal daily requirement of vitamin E. The rats were all maintained individually in wire cages with screen floors throughout the experiment. The reproductive performance was essentially the same in all three groups. Litters were limited to a maximum of six young. Six mothers whose lactation record was decidedly inferior were discarded. 90% of the 93 litters delivered by the remaining rats were successfully weaned. yielding 666 offspring with an average body weight of 42·5 g. at the 21st day of life. This is well above the minimum standard of 36 g. maintained in this laboratory for 21-day-old rats selected for studies relating to vitamin deficiency and reproductive performance. We have found that rats failing to meet this standard are decidedly inferior and frequently give very atypical results.

Throughout each lactation period the rats of the S and S + E groups were given diet 68 (Table II) and, to avoid too abrupt a dietary change, were also supplied with the regular diet until its removal on the 10th day of lactation. Those of the D + E group were deprived of the wheat germ oil supplement between the 10th and 21st days of lactation. By this means, the offspring of all three groups were denied any source of vitamin E except that obtained through placental transfer and through the mammary secretion.

It is of particular interest that the paralysis characteristic of the suckling young of mothers low in vitamin E [Evans & Burr, 1928] appeared in 20% of litters reared by mothers of the S group, but in none of the offspring of the S + E and D + E groups, indicating a significantly greater storage of vitamin E in the latter. It has since been found that this phenomenon rarely appears

when the vitamin E-deficient diet is substituted for the S diet at the 14th–15th days of lactation. We have failed to find in the paralysed rats, and in a large series of rats known to have been severely depleted of vitamin E from birth to adult life, the thyroid hypoplasia noted in such animals by Singer [1936] and Barrie [1937]; this confirms a recent report by Telford *el al.* [1938].

(*a*) *Placental and mammary transfer to female offspring.* Three groups of 92, 32 and 36 female rats selected respectively from the S, S + E and D + E mothers, were reared upon diet 68 and mated with normal males after they had attained body weights of approximately 150 g. Many began their pregnancies between the 30th and 40th days of experiment and, despite the occurrence of pseudo-pregnancy in 12 % of rats, all were successfully inseminated after an average of 45 days (i.e. at 66 days of age). In all 160 rats the first gestation terminated in complete resorption. Graphic representation of the average body growth of each group throughout the gestation-resorption indicated that the resorptive process was more severe in the offspring from mothers on the S diet than in those from mothers on the S + E and D + E diets, which is in accord with the small protection afforded to the testes of male offspring from the latter two groups compared with those from mothers on the S diet, as discussed below. Although we have failed to note any significant difference in the responses of offspring from mothers on the three types of breeding diets when used for the bio-assay of vitamin E concentrates, the data upon which strictly comparative estimates can be made are limited to 10 rats of each group and are considered insufficient to warrant a more positive statement.

(*b*) *Placental and mammary transfer to male offspring.* Three groups of 35, 21 and 21 male rats selected respectively from the S, S + E and D + E mothers, averaging 44·6 g. in body weight at the 21st day, were reared upon diet 68. A total of 95 testes and corresponding epididymides obtained either by operative removal or at autopsy after various intervals of feeding were weighed, prepared for histological study and classified according to the predominance of one or more of the five stages into which the degenerative process has been arbitrarily divided [Mason, 1926]. Special reference should be made to the value of histological examination of the epididymis in confirming and augmenting conclusions drawn regarding the previous and current alterations in the germinal epithelium of the testis. The results of this analysis are summarized in Table III.

It will be noted that in the offspring of mothers fed on the S diet (series S, Table III) early evidence of testicular injury appeared after as little as 30 days of feeding. At this time (51 days of age) mature spermatozoa were usually making their first appearance in the ducts of the caput epididymis both in the vitamin E-deficient rats and in litter-mate brothers reared from weaning upon the stock diet. Although the rats of this series had been receiving restricted amounts of vitamin E from birth, and some had spontaneously recovered from mild degrees of paralysis apparent during the last few days of lactation, the prepubertal growth and maturation of the testes exhibited no significant inhibition. Further investigations are being made in regard to the latter question, and to the earliest period at which the irreparable nature of the testicular injury can be demonstrated. The onset of testicular injury was approximately 10 days earlier than that observed in the earlier studies of Mason [1926] and from 5 to 10 days earlier than that noted by Ringsted [1936] in rats reared by mothers on a breeding diet containing a low but adequate content of vitamin E and subsequently fed on a vitamin E-deficient diet. The testicular damage observed in testes obtained after the same interval of feeding, and in the different seminiferous tubules of any one testis, was unusually uniform, especially when compared with the

Table III. *Extent to which placental and mammary transfer of vitamin E affects the onset of testicular degeneration in male rats reared on a vitamin E-deficient diet*

No. days on exp. after weaning	Series S		Series S + E		Series D + E	
	No. of testes	Stage of degeneration	No. of testes	Stage of degeneration	No. of testes	Stage of degeneration
30	5	N?-1	—	—	—	—
40	3	N?-1	—		—	—
	9	1	1	N?	1	N?
	6	1-2	—	—	—	—
50	1	1-2	—		3	N
	5	2-3-4	7	N-N?	3	N?-1
	5	3-4-5	2	N?-1	2	1-2
60		--	6	N?-1	3	N?-1
	1	3-4-5	3	1-2	3	1-2
	4	4-5	3	2-3-4	3	2-3-4
70	·	-	2	2-3-4	2	3-4-5
		—	4	3-4-5	4	4-5
80			2	4-5	2	4-5
Total no. of testes	39		30		26	

picture presented in testes of rats maintained for prolonged periods on vitamin E-deficient diets before the onset of sterility.

In the offspring of mothers fed on the S + E and D + E diets (series S + E and D + E, Table III) testicular degeneration appeared approximately 20 days later than in the S series described above, demonstrating an appreciable placental and mammary transfer of vitamin E when the daily intake of the mother was increased to 10 to 15 times the minimal. Preliminary attempts to measure the extent of this transfer, by feeding different amounts of a carefully assayed concentrate of vitamin E to 21-day-old rats treated in the same manner as those of the S series, indicated that the smallest amount of vitamin E necessary to delay the onset of testicular degeneration 20 days was approximately twice the single curative dose necessary for preventing resorption in female rats.

(*c*) *Placental versus mammary transfer.* The results of another series of experiments, which indicate that the mammary gland is almost entirely responsible for the transfer of vitamin E to the offspring, warrant brief mention. Three rats were given approximately 200 times the minimal single curative dose of vitamin E[1] during their period of gestation. Immediately after delivery, and before the young had opportunity to suckle, the litters were exchanged with those of recently delivered mothers fed on the stock diet. One other rat was given approximately 400 times the minimal single curative dose distributed over a period extending from the 13th day of pregnancy to the 7th day of lactation. The 22 male and 18 female offspring without access to any dietary source of E during lactation were reared upon the vitamin E-deficient diet.

In six male offspring whose storage of vitamin E was limited to that received during intra-uterine life, the time of onset of testicular degeneration was essentially the same as observed in the rats of series S (Table III). In 12 males suckled by mothers whose storage of the excess vitamin E administered during gestation apparently permitted close to the maximum mammary transfer of vitamin E to the adopted young, the testicular injury appeared about 20 days later, as noted in the rats of series S + E and D + E (Table III). Prolonged feeding of the

[1] In the form of a concentrate prepared from wheat germ oil by molecular distillation; kindly supplied by General Mills, Inc., Research Laboratories, Minneapolis, Minnesota.

vitamin E concentrate during lactation was deemed inadvisable because of possible contamination of the young with traces of the vitamin from the body or food of the mother. In the one instance where excess of vitamin E was given during late gestation and the first week of lactation, the four male offspring exhibited an additional delay of about 10 days in the onset of testicular degeneration, when compared with the latter group. It is particularly noteworthy that the 18 female rats derived from the seven litters just discussed, successfully impregnated at an average age of 64 days, invariably resorbed during their first gestation.

It is hoped that studies now in progress using a greater excess of vitamin E and a much larger series of animals will afford a more adequate measure of the maximum and relative efficiencies of the placental and mammary barriers in the transfer of vitamin E. The preliminary observations reported above merely suggest that the vitamin E storage of the offspring of mothers fed on the S + E and D + E diets represents close to the maximum attainable through combined placental and mammary transfer, that this storage is almost entirely due to mammary transmission of the vitamin and that increasing the daily vitamin E intake of the pregnant and lactating rats to several hundred times the minimal requirement does not permit enough vitamin E to pass the placental and mammary barriers to prevent resorption during the first gestation in female offspring otherwise deprived of vitamin E.

DISCUSSION

The results presented in this report are in close accord with other observations regarding the limited placental and mammary transfer of vitamin A [Dann, 1932; 1934; Baumann et al. 1934] and of vitamin D [McCollum et al. 1927] in the rat. Questions regarding storage capacity and excretion after administration of an excess of the fat-soluble vitamins, the "physiological" selectivity of the placental and mammary barriers and possible differences in storage capacity in the offspring during intra-uterine and postnatal life, are beyond the scope of the present discussion but offer interesting fields for further exploration.

The relative ineffectiveness of placental and mammary transfer of vitamin E in the rat, even when the maternal intake is exceedingly high, affords conclusive evidence that voluntary consumption of the stock diet by the young rat during the latter part of lactation is the primary factor responsible for the irregularity and delay frequently noted in the time of onset of sterility in vitamin E-deficient rats of both sexes. The complete elimination of initial fertility in female vitamin E-deficient rats through the simple expedient of substituting a vitamin E-deficient diet before the middle of lactation, with the production of offspring whose vitamin E reserves are very low from the time of birth and little influenced by minor variations in the vitamin E content of the maternal diet (which may be selected from the standpoint of cheapness and excellence for breeding purposes), should greatly facilitate the standardization of rats for the bio-assay of vitamin E and permit a much more accurate evaluation of assays conducted in different laboratories.

Furthermore, the established reliability of the first gestation period for assay tests (when properly controlled by the use of untreated litter-mate controls), which can be begun after an average of 45 days from the time of weaning, together with the economy of space and diet due to the smaller size of the test animals, are distinct advantages. The virginal state of the reproductive tract prior to the gestation used for the bio-assay test also appears appreciably to increase the

number of successful impregnations after positive matings [Bacharach *et al.* 1937] and should impart greater reliability to the assay procedure. We have obtained exceedingly uniform and satisfactory results in a series of more than 180 bio-assay tests conducted on rats prepared in the manner described.

In the present study, the male proved to be a more delicate indicator of vitamin E storage in the young rat than did the female. Certain problems relative to sex differences in storage capacity and vitamin requirement during pre-pubertal and post-pubertal life are being more thoroughly investigated in order better to evaluate the possible usefulness of the male rat for vitamin E assays.

SUMMARY

The variable responses of young rats of both sexes to vitamin E depletion can be closely correlated with the vitamin E content of the maternal diet to which the young have access after the adoption of free-eating habits during the latter part of lactation. By substitution of a vitamin E-deficient diet for the maternal diet during the last half of lactation, to limit the source of vitamin E to that obtained through the placenta and mammary gland, the initial storage of the vitamin in newly weaned offspring is decidedly limited and but little influenced by the vitamin E content of the maternal diet.

When the maternal diet contains but several times the minimal requirement of vitamin E, the vitamin E reserves of the offspring are negligible and, when reared upon vitamin E-deficient diets, males exhibit the onset of testicular degeneration between the 50th and 60th days of life, coincidently with the first appearance of spermatozoa. Increasing the vitamin E content of the maternal diet to about 15–20 times the minimal delays the onset of testicular injury about 20 days, which represents about the maximum protection obtainable through combined placental and mammary transfer, as indicated by preliminary tests in which the maternal intake was increased several hundredfold. Most of the vitamin transfer is accomplished through the mammary secretion.

Female rats whose initial vitamin E storage is limited to that obtained through placental and mammary transfer invariably resorb during their first gestation period when reared on vitamin E-deficient diets, even when the maternal intake is increased several hundredfold. The value of these observations in the improvement and standardization of methods for the bio-assay of vitamin E is emphasized.

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CCXXXIII. THE EFFECT OF ADRENALINE ON EMBRYONIC CHICK GLYCOGEN *IN VITRO* AS COMPARED WITH ITS EFFECT *IN VIVO*[1]

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MUCH less appears to be known of the mode of action of adrenaline on the breakdown of liver glycogen than of its mode of action on muscle glycogen. In muscle it has been shown that adrenaline initiates a breakdown of glycogen to lactic acid not only in an isolated perfused limb but in pieces of excised muscle *in vitro* [Hegnauer & Cori, 1934]. Lately Nachmansohn *et al.* [1936; 1937] have shown that adrenaline will act even on a muscle "brei", increasing the rate of formation of lactic acid and causing an accumulation of hexosemonophosphate, which seems to indicate that adrenaline may be acting here as a specific accelerator of one of the enzymes taking part in the phosphorylated glycogen glycolysis system in muscle.

With respect to its action on liver glycogen it is generally accepted that adrenaline produces a rise of blood sugar by breaking down preformed liver glycogen to glucose. But attempts to demonstrate the effect of adrenaline as a direct accelerating action on the liver glycogenase enzyme, converting glycogen into glucose, have so far proved unsuccessful [quoted Hodgson, 1936].

The various possibilities of the mode of action of adrenaline on liver glycogen and blood sugar seem to be:

(1) A direct action: (*a*) on the glycogenase enzyme independent of the cell structure, though this would appear unlikely in view of the experiments quoted by Hodgson; or (*b*) on the intact living liver cell, either breaking down a spatial separation of enzyme and substrate in the cell as Lesser [1920] suggested, or increasing the permeability of the cell wall to glucose, thereby causing a shift in the equilibrium between glycogen and glucose in the cell.

(2) An indirect action not demonstrable in the isolated living cell inasmuch as the final picture in the whole animal might be the resultant of a number of different actions of adrenaline. For example, the inhibition of glucose utilization under the influence of adrenaline reported by Cori & Cori [1928; 1929] and Colwell & Bright [1930] might account in part for the rise of blood sugar, while the constriction of the intrahepatic vessels as shown to occur with adrenaline by Clark [1928], Emery & Griffith [1931] and McMichael [1932] might be the cause of relative hepatic anaerobiosis and thus of the breakdown of liver glycogen.

In the hope of being able to throw some light on the question as to whether the action of adrenaline on liver glycogen is direct or indirect, an investigation was made of the effects of adrenaline and anaerobiosis on the glycogen content of cultures of embryonic chick liver where the conditions are not complicated by

the presence of nerves and blood vessels. In the present paper the results of this investigation are described, together with a study of the glycogen content of the liver of the embryo chick during development and a comparison of the effects of adrenaline on its liver and muscle glycogen.

METHODS

Tissue culture methods and histology. 11- to 16-day-old chick embryo liver was cultured in "hanging drop" cultures by the technique described by Doljansky [1929] with certain modifications. The tissue was cut into cubes about 1 μl. in size, which were planted on the surface of a firm plasma clot, containing 0·1 %, 1 % or 2 % sugar. The cultures were incubated at 38° with the liver tissue resting on the surface of the clot, and experiments performed on them after 4–6 days. By this time the cultures had grown out, partly by spreading and partly by cell division, into thin sheets of epithelial cells with a slight thickening at the centre, and their glycogen content could easily be seen by staining methods.

The glycogen content of the cultures was ascertained histologically in the following way: The cultures were stained first by the I_2 vapour method [Lewis, 1921] and the intensity and position of the glycogen staining noted; they were then fixed in 98 % alcohol and subsequently stained with Best's Carmine, haematoxylin or thionine being used as counter-stains. The agreement between the two different methods was satisfactory in all cases.

Reagents. All the adrenaline solutions used were made up from the pure crystalline solid (B.D.H. Epinephrine). Small quantities were weighed out and dissolved in an equivalent weight of N HCl, and diluted with Tyrode or Pannet and Compton solution immediately before using.

Glycogen determinations. Glycogen in embryo chick liver and muscle was determined by the method of Good *et al.* [1933], the sugar formed by hydrolysis being estimated by Somogyi's methods (Somogyi [1926]: for very small quantities of sugar, Somogyi [1937]). The liver and muscle samples were taken immediately after removing the embryo from the egg, and were dropped at once into weighed tubes of 30 % KOH.

RESULTS

1. *Growth and glycogen content of liver cultures.* Liver cultures were grown from 6- to 16-day-old chick embryos and in all cases abundant glycogen was found in the cells, both in the centre of the explant and in the cells at the edge of the outgrowing epithelial sheet. The cultures usually remained healthy and retained their glycogen for 5 to 7 days after implantation without the medium being renewed in any way. Nordmann [1929], who made an extensive study of the growth of liver cells *in vitro*, claimed that all glycogen was broken down during the implantation and was only built up again by the liver cells at the end of the first 24 hr. But glycogen was seen histologically 6 hr. and 19 hr. after implantation, and chemical estimations by Heatley's [1933] micro-method of pieces of embryonic liver, cut up to the size of culture implants and left to stand in saline for 1 hr., showed glycogen contents of 0·315 to 2·17 % (dry wt.).

The percentage of sugar in the medium did not have much effect on the glycogen content of the cultures, but those grown in a 0·5 % or 1·0 % sugar medium remained healthy and retained their glycogen longer than those grown in a medium to which no sugar was added.

For experiments with adrenaline, cultures of liver from 11- to 15-day-old embryos grown in a 0·5 % or 1·0 % sugar medium were used 4–6 days after implantation.

2. *Effect of adrenaline on liver cultures.* Groups of cultures were chosen as much alike as possible in size and extent of growth. To one group a drop of adrenaline solution was added, while to the control group a drop of Tyrode solution was added, and the cultures incubated at 38°. After varying periods of incubation they were tested histologically for glycogen. Solutions of adrenaline in concentrations of from 1 : 100 to 1 : 1,000,000 were employed and the pH of the solution was always adjusted to the same as that of the Tyrode solution used for the controls. The cultures were stained for glycogen after 5, 10, 15, 20, 30 and 40 min. and 7, 11 and 16 hr. The adrenaline-treated cultures, even those left for 16 hr. with a drop of 1 : 100 adrenaline solution, showed no signs of necrosis or destruction of the epithelial sheet, and in no case was their glycogen content diminished in comparison with the controls.

As Blaschko *et al.* [1937] and others have shown that liver tissue is one of the most active in destroying adrenaline in the presence of O_2, it was possible that, in spite of the strong doses used, the adrenaline was being oxidized to inactive compounds before it could diffuse into the cells. The drop of adrenaline was therefore sucked off and renewed every $\frac{1}{2}$ hr. for 3 hr., various concentrations of adrenaline being employed. There was no diminution of the glycogen content in the culture so treated, and it seemed unlikely that all the adrenaline added would have been oxidized before it could exert its effect.

In view of the possibility that in the absence of the pituitary the glycogenolytic action of adrenaline might be impared [Corkill *et al.* 1933; Cope & Marks, 1934; Bachman & Toby, 1936], an alkaline extract of ox anterior pituitary was prepared, according to the technique described by Cope & Marks [1934]. The fresh glands were ground up with acetone, the acetone removed by filtration and evaporation in air and the acetone-free tissue extracted with saline. This extract was added to cultures, both alone and together with adrenaline, in several doses for 40 min. No reduction in their glycogen contents, as compared with the controls, was seen histologically. In all about 50 cultures were treated with adrenaline under the various conditions described.

It seemed therefore that under the conditions of the experiments adrenaline had no direct effect on the glycogen of isolated liver cells.

There was, however, an important possibility to be investigated before these results could be taken as an indication that the glycogenolytic action of adrenaline was not a direct one. This possibility was that the young chick embryos from which these cultures were made might not respond to a dose of adrenaline *in vivo* with a fall of liver glycogen.

Vladimirov [1931] injected very large doses of adrenaline (1 ml. 1 : 100 in some cases) into the air sac of the hen's egg and obtained evidence that the blood sugar was raised in the 19- and 20-day, and occasionally in the 14- and 15-day embryo, though not in the embryo younger than 14 days. But the values for the blood sugar level of normal and adrenaline-treated embryos all varied enormously and the adrenaline was given usually in doses of 0·5-1 ml. The absorption of such quantities of fluid he reports earlier in the paper as causing a fall of blood sugar in the younger embryos, due to a dilution of the blood. This might easily conceal any slight rise of blood sugar that would be produced by the breakdown of liver glycogen in, say an 11-day embryo. In such an embryo the quantity of liver tissue, and therefore of liver glycogen, present is very small compared with the volume of blood circulating in the whole egg.

Estimations of the liver glycogen, rather than of the blood sugar, of such small embryos would therefore be more likely to reveal any glycogenolytic action which adrenaline might have.

Accordingly an investigation of the effect of adrenaline on the liver glycogen of chick embryos was undertaken, and at the same time its effect on muscle glycogen was studied.

3. *Effect of adrenaline on the glycogen of chick embryos.* As the literature contained no values for the liver glycogen content of the chick embryo earlier than the 14th day of incubation, estimations were first made of liver glycogen of normal embryos from the 8th to the 21st day (see Fig. 1). Appreciable quantities of glycogen are present from the 8th to the 12th day (0·2 to 0·9 %), followed by a sudden rise on the 13th or 14th day. The figures for each day of incubation show very wide variations, but the curve drawn through the average values shows the same rise up to the 19th day and subsequent fall at hatching as Vladimirov [1930] found.

The effect of adrenaline on the liver and muscle glycogen of 1-, 14- and 18-day-old embryos was then determined in the following way. A number of eggs, containing embryos of the same age, was divided into two groups, usually of about 6 to 10 eggs each. A small hole was made in the shell over the air sac, and with a hypodermic syringe 0·1 ml. 1:1000, and in a few cases 0·1 ml. 1:50,000 adrenaline was injected into the air sacs of one group, and 0·1 ml. Tyrode solution at the same pH was injected into the air sacs of the control group. The hole in the shell was sealed up carefully with paraffin wax and the eggs were incubated for 40 to 60 min. air sac uppermost. During this time all the injected solution was adsorbed and the surface of the air sac usually appeared white and dry. The

Fig. 1. Liver glycogen in chick embryos. Numbers denote the number of embryos used for a determination, if that number was greater than one.

embryos showed no ill effects after this treatment. They were removed from the eggs and their livers rapidly transferred to weighed tubes of 30 % KOH. Large strips of pectoral muscle were taken for the muscle glycogen determinations. In the case of the 18-day embryos samples from 1 or 2 chicks were used for each determination, in the case of 14-day embryos samples from 1 to 4 chicks, and in the case of 11-day embryos samples from 5 to 7 chicks. Altogether 174 chicks were used: 46 18-day, 67 14-day and 61 11-day.

As can be seen from Fig. 2, the glycogen contents of the livers of 11-, 14- and 18-day embryos treated with adrenaline were much reduced as compared with the saline-injected controls. It appeared therefore that adrenaline caused a mobilization of liver glycogen even in the youngest embryos from which liver cultures were made, and a lack of action *in vivo* could not therefore be the cause of the lack of action *in vitro*.

An investigation of the effect of adrenaline on embryonic muscle glycogen was of interest because of the findings of Needham & Nowinski [1937] that embryonic chick tissue was unable to glycolyse glycogen, being deficient, or partly so, in four of the essential enzyme components of phosphorylating glycolysis.

One of these enzymes was that responsible for the initial esterification of glycogen.

In Nachmansohn's [1937] experiments with frog muscle "brei" and adrenaline, and in Hegnauer & Cori's [1934] experiments with whole muscles placed in adrenaline solution, an accumulation of hexosemonophosphate was noted. It seems reasonable to suggest therefore that adrenaline might exert its accelerating effect at this initial step in lactic acid formation (*i.e.* the phosphorylation of glycogen). As slices of muscle from a 15-day chick embryo were unable to
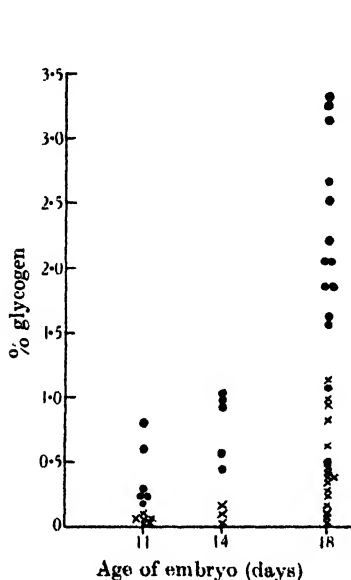
Fig. 2.   Fig. 3.

Fig. 2. Effect of adrenaline on embryonic chick liver glycogen.
● saline-injected embryos; × adrenaline-injected embryos.
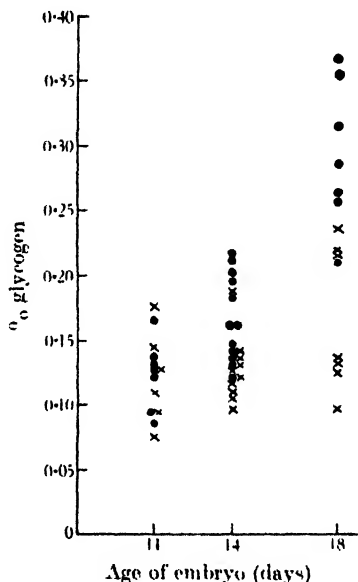
Fig. 3. Effect of adrenaline on embryonic chick muscle glycogen.
● saline-injected embryos; × adrenaline-injected embryos.

glycolyse glycogen in Needham & Nowinski's experiments, it might be anticipated that adrenaline would fail to cause a breakdown of muscle glycogen before the 15th day. Fig. 3 shows that no significant difference between the muscle glycogen values of the adrenaline-treated and saline-treated 11- and 14-day embryos was obtained, whereas on the 18th day of development there was a quite well defined reduction in muscle glycogen under the influence of adrenaline. No experiments have been done to ascertain whether a "brei" of 18-day embryo muscle can produce lactic acid from glycogen, but it would appear from these results that the enzyme, or enzymes, on which adrenaline acts to cause a breakdown of muscle glycogen to lactic acid are present by the 18th day of development.

In the 11- to 14-day embryo the liver glycogen, but not the muscle glycogen, is reduced by adrenaline.

The failure of adrenaline to cause any breakdown of glycogen in the cultures of 11- to 14-day embryonic liver was therefore in contrast to its effects *in vivo*. There might of course be some condition, not fulfilled in the culture experiments,

which would enable a glycogenolytic effect of adrenaline to be demonstrated *in vitro*, but in view of the other possibilities of adrenaline's mode of action on liver glycogen it seemed of importance to study the effect of anaerobiosis on the glycogen of liver cultures.

4. *The effect of anaerobiosis on liver cultures.* Cultures were subjected to an anaerobic atmosphere in special flasks as illustrated in Fig. 4. The coverslip on which the culture was grown was placed on the plate-glass sheet *P*, and held in
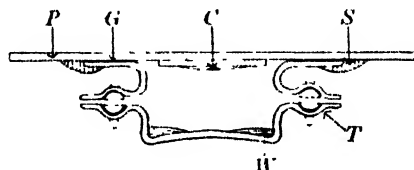
Fig. 4. Cross-section of apparatus employed for the subjection of cultures to an anaerobic atmosphere. *C* culture on coverslip. *G* - ground glass surfaces. *P* - plate-glass sheet. *S* - sealing compound. *T* - glass taps. *W* saline to keep atmosphere moist.

position with a little vaseline. A rubber grease was used between the ground-glass surfaces at *G*, and a sealing compound applied around the edges to complete the air-tight joint. The flasks were connected in series and a stream of N_2 and CO_2 mixture (95 % N_2 and 5 % CO_2) passed through them for 1 hr. The taps were then turned off and the flasks placed in the incubator. The culture could be observed microscopically at any stage of the procedure through the plate-glass sheet *P*.

Cultures were tested for glycogen after 1 to 6 hr. anaerobiosis at 38°. 10 hr. anaerobiosis were found to cause the death of all cultures so treated: the epithelial sheet appeared broken up and the individual cells rounded off. But cultures submitted to periods of 1–6 hr. anaerobiosis were found to remain healthy and to exhibit changes in their glycogen content.

Out of 45 cultures tested after 1–6 hr., 21 whose sister cultures all contained large quantities of glycogen were rendered completely glycogen-free.

DISCUSSION

The experiments reported here on embryonic chick muscle glycogen gave results which are in agreement with the work done by Needham and his collaborators. However, the results of the experiments which show a breakdown of glycogen in cultures of 11- to 14-day embryonic liver under anaerobiosis and a reduction in liver glycogen in the intact embryo's liver after injection of adrenaline are somewhat difficult to reconcile with their findings. They used "breis" of whole 3- to 13-day-old chick embryos and found no glycolysis of added glycogen, although lactic acid was formed rapidly from added glucose. From the results with tissue cultures and intact embryos reported in this paper it appears, however, that there is present in the liver, though not always in muscle, an enzyme system capable of breaking down glycogen. In order to explain the difference between these results and those of Needham *et al.* it must be assumed either (*a*) that this enzyme system can attack glycogen which is actually in the liver cells of the embryo but not that added to the surrounding medium, or (*b*) that the glycogen is breaking down to a substance which is neither glucose nor lactic acid. The former suggestion would seem the more likely.

The difference between the effect of adrenaline on the liver glycogen of chick embryos *in vivo* and *in vitro* appears to indicate that the glycogenolytic action of adrenaline is not a direct one on the liver cells but is dependent on the presence

of factors obtained only in the whole body. Against this assumption, however, is the possibility that liver cells in a culture may be in a somewhat different metabolic state from those in the intact embryo. For example, cultures which have had their medium repeatedly renewed over a period of about 14 days cease to store any glycogen, owing probably to an abnormally high rate of proliferation induced in them, as Doljansky [1930] suggests.

Although the cultures used in the experiments here reported still retained their glycogen and were capable of utilizing it under anaerobiosis, it is possible that even during their 4 or 5 days of life in an artificial medium the cells had become altered in some way which would affect the action of adrenaline. For instance, a certain definite reduction potential of the various cell components might be necessary to enable adrenaline to exert its effect, and a shift in this potential, due to a change in environment, might thus render it inactive as a glycogenolytic factor.

SUMMARY

1. Cultures of 11- to 14-day chick embryo liver, containing large quantities of glycogen easily observed by staining methods, were prepared. Adrenaline added to these cultures under a variety of conditions had no effect on their glycogen content.

2. Yet the chick embryo on the 11th, 14th and 18th days of incubation responded *in vivo* to doses of adrenaline with a well-marked fall of liver glycogen.

3. Although no reduction of muscle glycogen in the chick embryo under the influence of adrenaline was observed on the 11th and 14th days, there was a definite reduction on the 18th day of development. This result is in agreement with the results of Needham *et al.*, who found that the enzyme system in muscle, upon which adrenaline might exert its effect, was not present in the early stages of development.

4. Subjection of liver cultures to anaerobic conditions caused the complete breakdown of glycogen in a large number of cultures tested after 1–6 hr. anaerobiosis.

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CCXXXIV. THE INFLUENCE OF COBALT ON PINE DISEASE IN SHEEP

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PINE disease in sheep is prevalent in certain parts of the Cheviot Hills on both the Scottish and English sides of the border, and a preliminary paper by Greig *et al.* [1938] described the nature of the disease and its occurrence. Evidence was submitted to show that it is an anaemia, chlorotic in type, and that it may be controlled by the administration of Fe compounds.

Nearly 200 pining sheep on various farms in the area have been treated with iron ammonium citrate or crude iron oxide during the past 3 years, a course of treatment lasting about 3 weeks being sufficient to bring about recovery in 95% of the cases. These results confirmed the conclusion reached earlier that these Fe compounds were of direct curative value in combating the anaemia and restoring the sheep to health.

Fe compounds are also effective in preventing the disease. Hill sheep in pining areas readily acquire a taste for iron oxide put into boxes with salt, and on many farms where this practice has been adopted the disease has been almost entirely abolished. Ewes which have received the crude iron oxide have been kept experimentally for 2 years on severe pining land without a change of pasture and have remained quite free from the disease.

As previously reported, however, it was observed that although Fe therapy proved effective it was not always followed by an increase in the blood haemoglobin and, further, the composition of soils and herbage from the area lent support to the view that Fe itself was not responsible for controlling the disease. In his study of enzootic marasmus in Australia, Underwood [1934] showed that the affected sheep had apparently no difficulty in absorbing ample quantities of Fe from food, for there was a higher concentration than normal of Fe in the liver, kidneys and spleen, but they lacked something necessary for the utilization of the Fe. It was shown that this disease, which resembles pine, could be cured by an extract, free from Fe, of the limonite commonly used by farmers and it was suggested that the disease was due, therefore, not to a deficiency of Fe in the food but to some trace element or elements associated with the Fe.

In the present investigation, attention was directed to Cu of which a trace was present in the iron ammonium citrate, but treatment of pining animals in the field with a dilute solution of $CuSO_4$ was ineffective. This result was not unexpected, because the analyses of soils and herbage from the area did not reveal any correlation between disease and Cu content.

Soils and herbage. At the beginning of 1935 samples of soil and herbage were taken from eight localities in the area in question and fresh samples were obtained from the same places in July. Altogether 73 samples of soil, to a depth of about 4 in., and 90 samples of herbage, including grass, heather and hay, were analysed, and a summary of the results is given in Table I.

In work of this nature it has been usual for investigators to determine the constitutents in a strong HCl extract of the soil, but it was felt that a closer approximation to the material available to plant and animal would be obtained by preparing a less acid extract; the analyses were, therefore, carried out on the filtrates obtained by extracting 10 g. soil with 250 ml. ammonium oxalate-oxalic acid solution containing 200 mg. equivalents of $HC_2O_4^-$ per l. and of pH 3·2–3·3. It was also considered desirable to express the results in terms of volume rather than of weight of soil, on account of the variation in soil type encountered, so the apparent densities of the soils were determined and the results calculated to a volume basis. Samples of the herbage were ignited at a dull red heat and the ash was extracted with dilute HCl.

Fe was determined by means of thiolacetic acid and Cu with sodium diethyl-dithiocarbamate, both colorimetrically, using a Lange photocell comparator. There is an indication that both soils and herbage from the non-pining land at D are better supplied with Fe and Cu than those from the pining land at D but they are no better supplied than those from the pining area A. Determinations of Mn, by the periodate method were no more successful in throwing light on the problem and it appeared to be unlikely that pine, if it were a deficiency disease, was controlled by any of the three elements Fe, Cu or Mn. Attention was then directed to cobalt.

Cobalt as a factor. Marston [1935] and Lines [1935] had reported favourable results from the administration of minute quantities of Co to sheep suffering from "coast disease" in Southern Australia. This was followed by a paper by Underwood & Filmer [1935] in which Co was reported to give positive results in cases of enzootic marasmus. Then it was discovered that "bush sickness" in New Zealand could be successfully controlled, both in South Island and in North Island, by the use of Co; this work has been summarized by Hopkirk [1938]. In view of these results, an analysis of the crude oxide of iron used in the experiments in the Cheviot region was made, and this showed that Co was present to the extent of over 50 parts per million. It seemed probable, therefore, that the good results obtained from Fe compounds in the control of pine disease might similarly be due to the presence of Co.

Soils. Several publications on the relationship between sheep disease and Co have appeared in the last 2 years, and in a recent paper Kidson [1938], summarizing the available data on the Co content of soils, shows that figures varying between 0·0 and over 300 p.p.m. have been obtained. These are for concentrated HCl extracts and are therefore not strictly comparable with the results in Table I, but it would appear that the Border soils are generally very low in Co. The method of analysis [Kidson *et al.* 1936] is based upon the depth of orange colour formed by the reaction of Co with nitroso-*R*-salt and is subject to considerable error when the amount of Co in solution is of the order 0·001 mg., but it will be observed that the soils from Farm C and Lower land D (non-pining) contain more Co per unit volume than the others.

Herbage. The analyses of pastures in New Zealand [Askew & Dickson, 1937; Askew & Maunsell, 1937] have shown that the dry matter of unhealthy pastures usually contains less than 0·07 p.p.m. Co, whereas, for good quality pastures, the Co content is generally greater than 0·07 p.p.m. Paterson [1937], on the other hand, has recently suggested that 0·2 p.p.m. is insufficient and that 0·45 p.p.m. is sufficient in the pastures of Devon. The values given in Table I for the herbage lie between 0·20 p.p.m. and quantities less than 0·05 p.p.m. and those samples from C and D are rather better than the others. It would be quite impossible at present to draw a line showing the limiting value and there

Table I. *Average figures for minor elements in soils and herbage**

| | Soil | | | | | | | |
| | Spring | | | | Summer | | | |
Place	Fe	Cu	Mn	Co	Fe	Cu	Mn	Co
A. Pining farm, hill	92	29	80	1·2	193	48	147	—
Pining farm, field a	218	36	21	1·8	201	49	23	1·7
Pining farm, field b	222	48	47	2·0	145	32	34	2·0
B. Pining farm, peat	48	18	0·3	1·5	41	11	0·5	0·8
C. Non-pining farm, peat	141	36	2	2·8	68	16	0·8	0·8
Non-pining farm, mineral soil	248	34	64	5·4	519	59	108	3·7
D. Upper land (pining)	62	20	4	·1·7	70	23	7	1·6
Lower land (non-pining)	229	36	46	3·0	298·	43	20	3·7

| | Herbage | | | | | | | |
| | Spring | | | | Summer | | | |
	Fe	Cu	Mn	Co	Fe	Cu	Mn	Co
A. Pining farm, hill	18	49	116	0·15	7	42	69}	0·10
Pining farm, field a	—	—	—	—	8	44	91}	
Pining farm, field b	—	—	—	—	—	—	—	—
B. Pining farm, peat	12	24	54	Tr.	13	15	90	Tr.
C. Non-pining farm, peat	15	23	45	Tr.	13	16	56	Tr.
Non-pining farm, mineral soil	21	39	72	0·17	14	41	63	0·11
D. Upper land (pining)	20	54	33	0·17	6	35	51	0·07
Lower land (non-pining)	33	50	42	0·20	8	38	69	0·12

* Soils: Cu and Co p.p.m. by volume; Fe and Mn p./100,000 by volume.
Herbage: Cu and Co p.p.m. by weight; Fe and Mn p./100,000 by weight.
Tr. signifies less than 0·05 p.p.m.

is no definite relationship between soils and herbage. It is interesting to observe that if, as indicated by the New Zealand experiments, from 0·1 to 1·0 mg. Co per week is necessary to maintain health, a foodstuff containing only 0·1 p.p.m. cobalt (i.e. 1 mg. Co in 22 lb.) might be regarded as being adequately supplied with this minor element. Attention is directed to the fact, however, that there is a tendency, as shown by Askew & Maunsell [1937], for the Co content to fall during the growing season, so that individual results may be liable to misinterpretation. It was obviously necessary to test whether Co was the limiting factor in the health of the sheep by administering a supplement of Co to affected animals.

Preliminary field tests with cobalt. In May 1937 four pining Cheviot ewes were confined on a piece of pining hill land which had been found by previous tests to accentuate the trouble in a very marked degree. The ewes were given doses of 3 mg. Co per head in the form of $CoCl_2$ (Analar) in distilled water. This dosage was given daily for 1 week, and thereafter every second day, a total of 12 doses being administered to each sheep. Within 10 days an improvement in the appearance and vigour of the sheep was noted, and after 17 days recovery was regarded as being complete. All four ewes nursed lambs throughout the test and these also improved along with their mothers. The ewes increased, on the average, by 7·87 lb. live weight per head in 3 weeks from the date of commencement of the test. They were thereafter returned to their hill grazings and continued to make good progress throughout the summer.

In June 1937 a further three pining ewes were treated on similar lines and these also made a rapid recovery. They increased in live weight, on the average, by

8·56 lb. per head in 3 weeks. In 6 weeks the increase amounted to 15·4 lb. per head.

As 3 mg. Co per day had proved effective in the above instances it was decided to try smaller doses. A test was made on a pining farm in a different part of the Cheviot area during August, 1937. Five pining hoggs and one pining ewe were treated with 1 mg. Co per head per day. Within 3 weeks they had lost all symptoms of the disease and were considered to be effectively cured.

As these preliminary tests had proved successful it was arranged to carry out a properly controlled experiment.

Controlled experiments with cobalt. Twenty Cheviot weaned lambs, or hoggs, which were healthy and free from pining, were divided into two groups on 16 August 1937, and enclosed on a section of pining hill land at an elevation of 800 ft. Both groups ran together and received no feeding except grass. An experiment on the same land during the previous season had shown an incidence of pining to the extent of 73 % within 2 months. The conditions were, therefore, favourable to the manifestation of the disease. The treatment given was as follows:

Group 1. 10 hoggs; 1 mg. Co per head per day for 14 days.
Group 2. 10 hoggs; no treatment.

Co was given in the form of $CoCl_2$ (Analar) dissolved in distilled water. The animals were dosed individually, the doses being spread over a period of 3 weeks, this being equivalent to the length of time normally occupied by a change off pining land.

The treated hoggs made good progress and remained entirely healthy. The untreated hoggs, on the other hand, soon began to develop symptoms of the disease. While all were healthy on 16 August, 8 out of 10 were pining by 11 September, and by the end of September only one hogg remained in a healthy state. The average live weights of the hoggs were as follows:

	16 August lb.	5 October lb.	Increase lb.
Treated group	38·35	46·25	7·9
Untreated group	36·4	39	2·6

The treated group gained 7·9 lb. live weight per head over the period, while the untreated group only gained 2·6 lb. There was an increase in the colour of the conjunctiva of the treated group, as ascertained by comparisons with a Tallqvist haemoglobin scale, while in the case of the untreated group, there was a decrease in the colour which was found on the average to amount to 9 %. It was clearly demonstrated by this test that the administration of 1 mg. Co per day for 14 days had entirely prevented the onset of pining.

The procedure next followed was to dose the untreated group, all of which, except one, were now affected with the disease. It was decided to attempt to cure the disease by doses of 0·5 mg. Co per head per day. Accordingly, 14 doses were given at this rate, spread over 3 weeks, commencing on 5 October. By 21 October quite a marked improvement was noted in the majority of the group, and within 1 month they had all recovered except two which had been particularly badly affected. These were given five additional doses of 3 mg. Co per day, after which recovery was rapid.

All the hoggs continued to run on the same section of pining land till 9 December and received no hand feeding of any description. Owing to heavy snow and frost thereafter, however, it was necessary to feed hay, the produce of

pining land. This treatment continued till 15 February, after which concentrates were fed and the experiment was terminated.

The original pining group increased in live weight by 3·2 lb. per head between 5 October and 9 December and, considering the severe conditions at this altitude of 800 ft., this afforded good proof of their recovered vitality.

While this experiment was in progress an outbreak of pining occurred among ewes and hoggs on the same hill farm, 30 animals being affected. Treatment was administered by giving for 3 weeks 1 mg. Co per day mixed with salt, together with a little concentrated food. As some of the sheep would not eat this mixture, they were given doses in liquid form. Recovery took place in all cases.

DISCUSSION OF RESULTS

The influence of Co in animal nutrition has been a subject of investigation by several workers. Waltner & Waltner [1929] found that the addition of Co to the diet of rats produced a marked increase in red blood cells. A similar result was noted in dogs by Mascherpa [1930], and other workers have confirmed these results. Beard & Myers [1929] found that Co was a useful supplement in curing anaemia in rats produced by feeding them with cow's milk, but Cunningham [1931] doubted whether a true anaemia was developed in rats fed on this diet.

Dixon [1937] showed that a weekly drench containing 0·8 mg. Co gave a good response in the control of bush sickness, and the effect was augmented by inclusion of 0·18 mg. Ni later in the treatment, when presumably the "primary deficiency of cobalt was satisfied". Filmer & Underwood [1937] found that 0·1 mg. Co per day was effective in controlling enzootic marasmus in Western Australia, whilst Marston *et al.* [1938] have shown how Cu, although useless alone, has a marked effect when administered along with Co in the treatment of coast disease of Southern Australia.

In the present investigation it has been shown by analysis of herbage that the amount of Co available to sheep on pining land is very small. The administration of 14 mg. of Co per sheep, however, entirely prevented the disease for 6 months on severe pining land. The consumption of Co over the whole of this period was less than 0·1 mg. per day.

Regarding therapeutic results from Co, a cure was repeatedly effected in the experiments described above by giving 14 doses of 1 mg. each, spread over a period of 3 weeks and the cure remained effective for many months. Attempts to deal with the disease with 0·5 mg. doses were only successful where pining was in its early stages.

It may be concluded from these experiments, therefore, that for preventive and curative purposes 1 mg. doses of Co are necessary to ensure reliable results in the treatment of pine disease in sheep, and that a minimum of 14 doses is desirable. The cost of the material is only about one penny for 30 sheep. These results have been obtained by administering the Co in solution to the animals individually, but the most practicable method of treating hill sheep is to allow the stock access to a mineral mixture containing an adequate amount of $CoCl_2$. The whole of the stock on a pining farm were treated in this manner, during the winter and spring of 1937–38, with complete success. The application of $CoCl_2$ mixed with fertiliser or sand to pining land merits consideration, however, in some of the more accessible places.

SUMMARY

1. Pine disease in sheep in the Cheviot region of Scotland is a nutritional anaemia which can be cured and prevented by the administration of cobalt in the form of cobalt chloride.

2. The disease is not due to a deficiency of Fe or Cu or Mn.

3. The beneficial results previously obtained from the feeding of Fe compounds may be attributed to the presence of Co in the Fe compounds used.

4. The administration of 1 mg. Co per day for 14 days is sufficient to prevent the disease on severe pining land for a period of 6 months. A similar quantity is effective as a cure.

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CCXXXV. STUDIES ON DIFFUSING FACTORS. ACTIVE PREPARATIONS FROM MAMMALIAN TESTICLE AND THEIR BIOLOGICAL ASSAY

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AQUEOUS extracts of mammalian testicles contain a factor which dramatically increases the permeability of the skin to injected fluids and particles [McClean, 1930; 1931; Hoffman and Duran-Reynals, 1931]. An intracutaneous injection of saline, serum or an extract of any organ other than testicle produces a well-marked bleb, the margins of which remain quite distinct for 20–30 min. A similar injection of testicular extract, on the contrary, immediately diffuses into the dermis so that after 30–60 sec. it is difficult to identify the site of injection.

It can be shown that this diffusing factor is active in very low concentrations in the following way. Ascending dilutions of the extract are mixed with a constant amount of indian ink or a solution of diphtheria toxin, and the mixture is injected intracutaneously into the shaved back of a rabbit. Control injections of indian ink or toxin mixed with saline are also made. It is then observed that the area over which the particles of indian ink spread, or the size of the cutaneous lesions due to diphtheria toxin, is increased by high dilutions of testicular extract.

The factor responsible for the effect is associated with the germinal epithelium of the testicle, and is also found in extracts of spermatozoa [McClean, 1931]. Subsequent work has revealed that factors with similar diffusing properties can be obtained from the most diverse sources; e.g. from filtrates of invasive strains of staphylococcus and streptococcus [Duran-Reynals, 1933], from organisms of the gas-gangrene group and virulent pneumococci [McClean, 1936], from extracts of malignant tissues [Boyland & McClean, 1935], from snake and spider venoms [Duran-Reynals, 1936] and from leech extracts [Claude, 1937]. It appears therefore that these diffusing factors may have considerable physiological and pathological importance, and their chemical isolation is desirable.

A method of purification of the diffusing factor from bull's testicles has been described by Morgan & McClean [1932]. They estimated the activities of different fractions by determining the highest dilution which would produce an increase of at least 20% in the area of the cutaneous lesion produced by a standard dose of diphtheria toxin. This method is not sufficiently reliable for quantitative assay of diffusing factors for the following reasons: the skins of the experimental animals vary in their susceptibility to the toxin and to the diffusing factor; no standard diffusing preparation is used against which samples of unknown potency can be compared in each animal; the time of evolution and the type of the toxin lesion varies in different animals, and therefore the area of the lesions is not always clearly circumscribed and cannot always be determined at the same interval after injection; it is quite impossible to distinguish between successive tenfold dilutions of a given solution, and it is doubtful whether hundredfold dilutions can be consistently differentiated.

For further purification of the diffusing factor a more accurate method of titration is required. The use of indian ink as an indicator for the quantitative estimation of purified samples has been criticised by Favilli & McClean [1934]. They found that particles of indian ink adsorb the partially purified diffusing factor and that the amount adsorbed varies in different preparations. Furthermore, when indian ink is used it usually remains accumulated at the site of injection, and only a small amount spreads round it forming a halo of faintly stained tissue. The boundaries of this halo are not sharp enough to allow accurate measurements. The ideal indicator would be a soluble colouring matter which is not absorbed by the skin tissues, which spreads uniformly but not excessively, and is not removed too quickly. Such properties are possessed in large measure by haemoglobin. During the first few hours following an intracutaneous injection of a solution of a foreign haemoglobin the deeply pigmented spot at the site of injection is surrounded by a halo of less pigmented tissue. The outer boundaries of this halo are sharp and can be accurately measured. After 2–3 hr. the haemoglobin is evenly distributed over the whole area, which is approximately elliptical in shape. The stained areas produced by intracutaneous injection into different rabbits of a fixed amount of haemoglobin solution containing a given concentration of diffusing factor are relatively constant in size. Thus if the same titration is repeated in several animals the average of the results will not be influenced by extreme values obtained in a particular rabbit.

The haemoglobin does not disappear from the skin for at least 30 hr. The rate of increase of the coloured areas during this time can be determined. It has been found that the area coloured by haemoglobin increases rapidly during the first hour, the rate of increase being proportional to the concentration of diffusing factor. After 2–3 hr. it becomes independent of concentration (Fig. 1). The area of a coloured patch produced by a certain concentration of diffusing factor is always larger than that corresponding to an injection of a hundredfold dilution. The results are reproducible if similar injections are made in different places on the back of the same rabbit. If the results obtained in three rabbits are averaged it is possible to distinguish between tenfold dilutions. The experimental error is too large to allow closer titrations (Fig. 2).

The following standard procedure was followed throughout this work.

To compare the activities of two preparations of diffusing factor a series of tenfold dilutions of each is prepared. An isotonic solution of haemoglobin is used as a diluent. Intracutaneous injections of each concentration of one preparation are made in one side of the shaved back of each of three rabbits. In the other flank injections of the corresponding dilutions of the second preparation are made. Dilutions containing the same concentration should give coloured patches of similar area and moreover their rates of spread should be the same (Fig. 3).

For testing solutions of unknown activity, comparison is made with a standard stable preparation of diffusing factor, stored in the form of a water-soluble powder. The diffusing activity of this standard has been determined by establishing the smallest concentration at which it produces a definite increase in the rate of diffusion of haemoglobin and in the area of tissue over which it spreads. Since the susceptibility of the skin of individual rabbits to the diffusing factor may vary somewhat, determinations were made on five animals and the mean of the results was taken as a measure of the diffusing potency of this preparation (Fig. 4).

A considerable concentration of the diffusing factor present in aqueous extracts of bull testicles has been achieved by Morgan & McClean [1932]. They
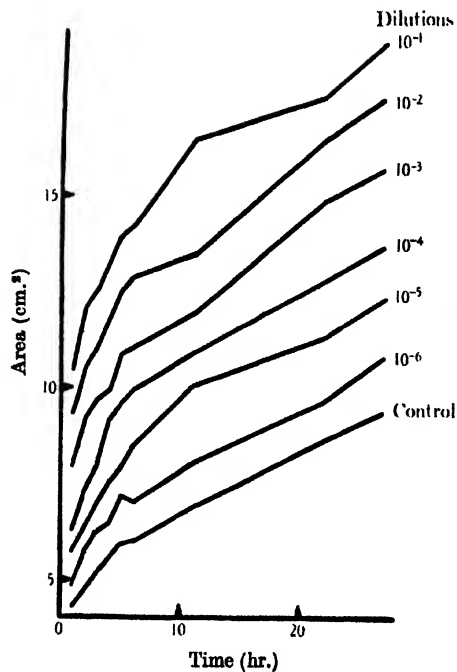
Fig. 1.
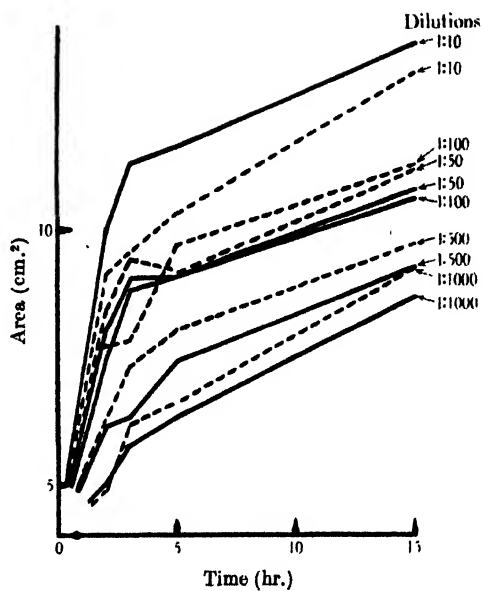
Fig. 2. —— Right flank. --- Left flank.
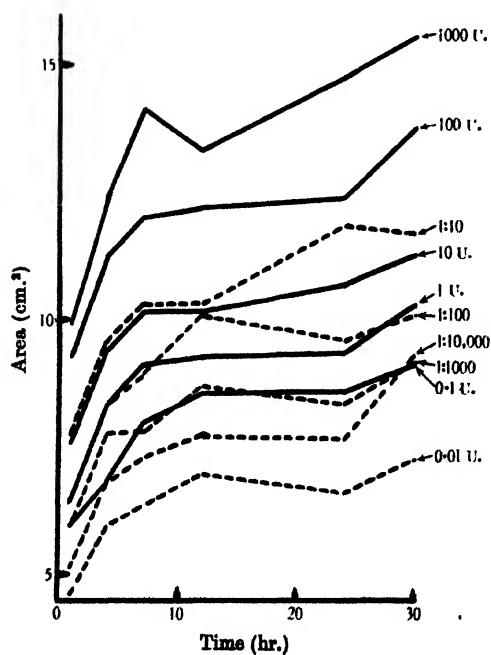
Fig. 3. —— Standard. --- Unknown.
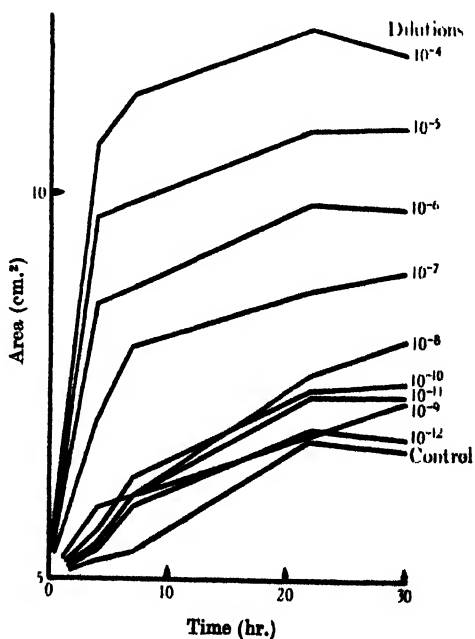
Fig. 4.

found that most of the protein could be removed from such extracts by precipitation with neutral lead acetate. From the filtrate the diffusing factor, together with $Pb(OH)_2$, was precipitated on making alkaline. The active material could also be precipitated with basic lead acetate. Claude & Duran-Reynals [1937] have described another method of concentration. This method involves treatment of crude testicular extract with acetone and fractionation of the redissolved acetone precipitate with $(NH_4)SO_4$. The active material precipitates with fully saturated but not with half saturated $(NH_4)_2SO_4$. Preparations obtained in this way obviously contain large amounts of protein, and, since Claude & Duran-Reynals did not employ a quantitative method of assaying biological activity, it is difficult to form any idea of the degree of purification attained by them.

Preparations containing relatively little protein have now been obtained by a method similar to that used by Morgan & McClean, save that the basic lead precipitate was decomposed with H_2S and the active material precipitated from the Pb-free solution with acetone. In agreement with the results of earlier workers, the diffusing factor was found to be non-dialysable (cellophane membranes) and insoluble in non-aqueous solvents with the exception of glacial acetic acid. Readily soluble in 50 % alcohol, it is insoluble at concentrations of alcohol greater than 70–75 %. The failure to dialyse must be regarded as a well established fact since as little as 0·01 μg. of active material per ml. can be detected biologically. This might indicate that the substance causing increase in dermal permeability has a high mol. wt.

The results of ultracentrifugal sedimentation, on the other hand, seem to indicate a relatively low mol. wt. Dr A. S. McFarlane (of this Institute), to whom my thanks are due, carried out an experiment in which a 2 % solution ($pH = 6·2$) of an active material (1000 units per ml.) was centrifuged. The density of this preparation was measured pyknometrically at 25˙ and found to be 1·40. Very little sedimentation was observed after 3 hr. in a field 300,000 times gravity. The degree of sedimentation observed was such that the mean mol. wt. could not be greater than 10,000. The boundary was not sharp.

Some of the effects of purified preparations of diffusing factor on compressed unimolecular films were studied by Dr J. Schulman (Cambridge) to whom I also wish to express my thanks. The surface potential of a gliadin monolayer was substantially changed on adding the preparation. The alteration observed was of nearly the same order as that produced by a similar concentration of saponin. Unlike saponin, however, the tested preparation had no effect on the surface potential of a cholesterol monolayer.

The stability of purified concentrates of diffusing factor in solution at different pH values has been studied. At pH 3 complete loss of activity occurred after 24 hr. at room temperature. No loss of activity occurred at pH 4, 5, 6, 8 or 9. At pH 11·5 noticeable destruction (about 90%) was observed, while activity was completely destroyed after 24 hr. at $pH < 3$ or $> 11·5$. Dilute solutions (0·1 %, pH 7) of the purest preparations available lost their activity rather easily on heating. At temperatures of 60° and over, more than 90% of the activity was lost in 5 min. At 37° the solutions were much more stable, more than 90% of the activity being retained after 24 hr.

These results appear to be at variance with those of Claude & Duran-Reynals [1937] and Aylward [1937] who found some of their preparations to be relatively stable. The fact that these workers did not use a quantitative assay method may explain their results, since large changes in the concentration of diffusing factor solutions cause relatively little change in the area over which haemoglobin spreads in experimental animals.

The purest preparations of diffusing factor so far obtained are obviously mixtures of different substances. The only pure compound so far isolated from them is *meso*-inositol. This substance crystallises on addition of alcohol to dilute aqueous solutions of purified preparations. It can also be isolated from them by vacuum sublimation. Neither inositol nor its phosphate, phytin, show any diffusing properties in biological tests.

<div align="center">EXPERIMENTAL</div>

Isotonic solution of haemoglobin. A measured amount of defibrinated sheep's blood is centrifuged and the serum decanted off. After washing with isotonic saline the erythrocytes are lysed with distilled water and made up to the original volume of blood. To render this solution isotonic 0·1 vol. of 8·5 % NaCl is added. A solution prepared in this way is used throughout as a diluent for preparing dilutions of the material to be tested.

Injections. Young male rabbits with unpigmented skin are used throughout. Rabbits with coloured fur are preferable. The back and flanks of the animal are shaved with electric clippers.

The rabbits are gripped as for measuring the coloured areas (see below). A line is drawn along the backbone of the animal and each flank divided into a number of squares corresponding to the number of injections to be made. The length of back generally available is 15-20 cm. and the areas coloured by the haemoglobin have a diameter of 2-5 cm. Therefore only five injections can be made on each flank. In order to use most of the available space the injections on each flank are made alternately at 1 and 2 cm. from the backbone. Only one row of injections can be made on each side of the rabbit.

The material is injected intracutaneously and deeply coloured weals result. When high concentrations of diffusing factor are injected the weals flatten almost immediately and the haemoglobin spreads rapidly over a large area of skin. 0·3 ml. is injected into each site; smaller amounts give areas difficult to measure accurately, and when larger volumes are injected, the injected fluid is apt to leak back through the point of injection.

Measurement of the coloured areas. The rabbits are gripped by the ears and the upper part of the hind legs and held on the knees of a seated assistant. Unnecessary strain of the animal's body is to be avoided. The longest (D) and shortest (d) axes of every coloured area are measured ($\pm$ 1 mm.) with calipers. The area (S) is calculated assuming that the coloured patches are regular ellipses $\left(S = \pi \dfrac{D.d}{4}\right)$. Variations due to the position in which the rabbits are held can be partially avoided by releasing the rabbit and repeating the reading after a short time. The average of three consecutive readings is taken as the area of the stain.

Less than 5 min. are required to measure the areas on each rabbit. During the first hour following injection the coloured areas grow too rapidly to allow three successive accurate readings to be taken but during the later measurements the area does not alter appreciably in the time required.

Influence of the concentration of diffusing factor on the rate of spread of haemoglobin through the dermal tissues. From an aqueous solution (10 mg. per ml.) of a dry preparation of diffusing factor a series of tenfold dilutions was prepared, the diluent being in each case an isotonic solution of haemoglobin. Dilutions ranging from 10^{-1} to 10^{-6} were injected into the flanks of three rabbits. Two controls of haemoglobin alone were also injected. The average rate of spread of the different coloured areas is shown in Fig. 1. The area of the lesions is consistently proportional to the concentration of diffusing factor.

Reproducibility of the values. Into one flank of each of three rabbits a series of fivefold dilutions (in isotonic haemoglobin) of a solution containing the active material was injected. The same dilutions were injected into the opposite flank of the same rabbits. The rate of spread of the pigmented areas is shown in Fig. 2. The lines representing consecutive fivefold dilutions are not constantly arranged according to their concentration. When three rabbits are used the experimental error does not allow one to distinguish between fivefold dilutions. The limit of accuracy of this method appears to be tenfold dilutions.

The haemoglobin disappears from the skin in about 3 days. Identical injections were repeated after an interval of 4 days in the same areas of the animals which had been used in the previous experiment. The area and rate of spread of the coloured areas were much smaller and less regular than after the first injections. It is therefore inadvisable to use the same rabbits twice.

Activity of the standard preparation. A dry preparation obtained by a method similar to that described by Morgan & McClean [1932] is used as a standard. Serial tenfold dilutions of a solution (1 mg./ml.) of this preparation were made. Nine consecutive dilutions and a control of haemoglobin solution alone were injected into the flanks of a group of five rabbits. The lowest dilution contained 10^{-4} g. ml. and the highest 10^{-12} g. ml.

An average of the results obtained in the five animals was plotted graphically (Fig. 3). It will be seen that no definite diffusion was shown by solutions containing less than 10^{-8} g. ml. 0.01 μg. is the minimal diffusing dose of the standard preparation. 1 μg. of this powder is considered as a unit, and the activity of any preparation expressed in units per mg. or ml. Thus the standard preparation has one thousand units per mg.

Titration of a solution of unknown activity. Injections of a series of five successive tenfold dilutions of a solution of the standard preparation were made in one flank of each of a group of three rabbits. The strongest solution contained one thousand units per ml. (1 mg./ml.). Tenfold dilutions of the solution of unknown activity were injected into the other flank of the rabbits. The results are shown in Fig. 4. Dilutions of 10^{-1}, and 10^{-2} and 10^{-3} of the unknown solution produced coloured areas of similar size and rate of spread to those produced by the standard containing 10, 1 and 0.1 units per ml. Thus the diffusing activity of the solution under test was equivalent to 100 units per ml.

Concentration of the diffusing factor. The starting material used was either the testicles of recently killed bulls or a desiccated bull's testicle powder supplied by the Instituto Biologico Argentino, Buenos Aires.

Twenty-four fresh bulls' testicles (10 kg.), freed from the adjacent membranes, were minced in a meat mincer and the resulting pulp extracted with water (10 l.). Alternatively the dry testicle powder (1.5 kg.) was directly extracted with water (10 l.). After standing in the cold room ($+5°$) for 24 hr. the water was decanted off and the residue re-extracted three times more in a similar way.

A saturated solution of neutral lead acetate (1 l.) was added to the combined crude extract (40 l.) and the inactive precipitate removed. To the clear filtrate more neutral lead acetate (500 ml.) was added and then the solution made alkaline (pH 8.9) with ammonia. The active precipitate was collected. It was suspended in water (1 l.) and acidified (pH 4) with acetic acid. After removing the insoluble residue the Pb was precipitated by H_2S. When precipitation was complete the mixture was filtered and the filtrate concentrated *in vacuo* (bath temperature 37–40°) to half its original volume (500 ml.). Addition of acetone (4 l.) precipitates the active material quantitatively. The precipitate was extracted with water (50 ml.) and reprecipitated with acetone (500 ml.), the active

constituents now separating as a thick oil. This was almost completely soluble in water (50 ml.) and after removing a small amount of insoluble material the active constituents were precipitated as a white amorphous powder (4 g.) by addition of alcohol (250 ml.).

On account of the inaccuracy of the biological assay, even with the improvements described in this paper, it is difficult to obtain a precise idea of the concentration reached. The starting material (1500 g. of dry testicle powder) had some 10 units per mg. and the purified powder had 1000.

Purified preparations of diffusing factor are very readily soluble in water and slightly hygroscopic. They give a positive biuret reaction and a precipitate with trichloroacetic acid. The average percentage composition of the concentrate is C, 37·5%; H, 6·5%; N, 6·6%; O. (by difference) 58·4%; P, 2% and 9% ash. They are completely soluble in 50% alcohol and partially in 70%, in the latter case only traces of diffusing activity going into solution. Soluble in glacial acetic acid, they are insoluble in all other non-aqueous solvents tried.

By addition of alcohol to a dilute solution of purified diffusing factor and keeping the solution for 3 months at room temperature an inactive crystalline material is obtained. The same inactive substance could be isolated from active preparations by sublimation *in vacuo* (200–220°/0·5 mm.). Recrystallised by adding an excess of alcohol to a concentrated aqueous solution or from hot 50% acetic acid it had M.P. 222–225°. (Found: C, 39·7: H, 6·8; $C_6H_{12}O_6$ requires C, 40·0; H. 6·7.) The substance showed all recorded properties of *mesoinositol* and a mixed M.P. with an authentic specimen of this substance gave no depression.

Effect of the pH on the activity of solutions of purified preparations of diffusing factor. A solution (2 mg./ml.) of a purified preparation of diffusing factor (1000 units per mg.) was distributed in a series of tubes and each sample diluted with an equal volume of a 0·2 M buffer solution of the required pH. After 16 hr. at room temperature a small amount of each sample (2 ml.) was buffered with a 0·15 M phosphate buffer (pH 7·2, 8 ml.) and the resulting solution diluted ten times with isotonic solution of haemoglobin.

Into one flank of each of three rabbits the solutions which had been in acid medium were injected. A further three rabbits were used for the solutions which had been in an alkaline medium. The other flanks of the rabbits were injected with dilutions of the standard preparation having 100, 10, 1 and 0·1 units per ml. Control injections of haemoglobin alone were also made.

The rates of spread of haemoglobin injected with the solutions of diffusing factor which had been kept at pH 2, 3 and 14 were almost identical with that of the control injection of haemoglobin alone. The rate of spread of the one kept at pH 11·5 was similar to the one of the standard having 1 unit per ml. At pH 4, 5, 6, 8, 9 and 10 no destruction of activity was noticed, the haemoglobin spreading at the same rate as the solution of the standard preparation having 10 units per ml.

Effect of heat on the activity of solutions of purified preparations of diffusing factor. Two samples of a solution (1 mg./ml.) of a purified preparation (1000 units per mg.) of diffusing factor were heated at 60° and 80° respectively. Samples were taken out at 5, 15, 30 and 100 min. After cooling them as quickly as possible they were kept about 20 hr. in the cold room (+5°). Each sample was diluted ten times with an isotonic solution of haemoglobin and injected into one flank of two groups of three rabbits, each group corresponding to one temperature. The other flank of each rabbit was injected with dilutions of the standard preparation.

The rate of spread produced by the sample heated 5 min. at 80° indicated an activity corresponding to 0·1 unit per ml., whereas the unheated injected solution corresponded to 100 units per ml. Although 99·9 % of the activity was lost on heating the increase in the rate of spread of the area coloured by haemoglobin produced by the heated solution was nearly half of the increase produced by the unheated one which was a thousand times more active. 99 % of the activity of the sample heated at 60° was lost in 5 min. and about 99·9 % in 15 min.

Another sample of a similar solution (1 mg./ml.) of the same preparation (1000 units per mg.) of diffusing factor was kept in an incubator (37°) for one day. The activity of this heated solution was found to be nearly the same as that of the standard preparation (1000 units per mg.).

SUMMARY

1. A new procedure is described for the biological assay of preparations of diffusing factor from mammalian testicle. This method, in which haemoglobin is used as an indicator, enables successive tenfold dilutions of diffusing factor to be differentiated with certainty.

2. An improved method for concentrating the diffusing factor is described, and preparations have been obtained. 0·01 μg. of which caused marked increase in tissue permeability.

3. From highly active preparations *meso*inositol has been isolated; it has no diffusing activity when tested biologically.

4. The activity of concentrates of diffusing factor is rapidly destroyed at temperatures above 37° and at pH below 4 or above 10.

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CCXXXVI. ELECTROPHORESIS OF PEPSIN

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THE first investigation of the behaviour of pepsin in an electric field was published by Michaelis & Davidsohn [1910]. They attempted to determine the sign of the charge on commercial pepsin ("purissimum Grübler") in the pH range 1·0–4·3, using the well-known Michaelis transference apparatus. After passing current through the apparatus, qualitative tests were made for pepsin in the compartments above the stopcocks. They found that pepsin is negatively charged at pH > 3·7, positively charged at pH < 2·0, and within this interval shows migration towards both sides. This latter phenomenon was also observed at very acid reaction (0·1 N HCl). The isoelectric point, obtained by taking the average of the hydrogen ion concentrations corresponding to the pH limits mentioned, is given as cH^+ $5·5 \times 10^{-3}$, or pH = 2·25.

Since, according to later investigations of the electrophoresis of proteins, mobility is a linear function of pH and not of cH^+ in the neighbourhood of the isoelectric point, it would seem preferable to take the average of the pH values, which would give pH 2·85 for the isoelectric point.

Ringer [1915], using a similar apparatus but a purified pepsin preparation, obtained somewhat different results: the enzyme showed anodic migration even in the most acid solutions and consequently had no isoelectric point. Addition of protein decomposition products (pepton Grübler), however, caused a partial cathodic migration in acid solution, which was interpreted as due to partial combination with the enzyme. On the other hand the electrochemical properties of crystalline pepsin, first prepared by Northrop, again indicate an isoelectric point of 2·8 (solubility minimum, titration curve, electrophoresis of particles of finely ground crystals) according to observations by Northrop himself [1930]. Ågren & Hammarsten [1937] in a recent paper made some interesting measurements on the electrophoresis of solutions of a crystalline pepsin preparation in the apparatus of H. Theorell. At pH 2·27 and 2·30 they found positive migration, at 3·34 negative, which would also indicate an isoelectric point around 2·8. A certain separation of peptic activity and total nitrogen was observed at pH 3·34, but at more acid reaction both migrated together. However, in recent measurements with a different material Ågren found only negative migration for pepsin (private communication).

The question of the electrophoretic homogeneity of pepsin was dealt with in considerable detail already by Ringer in his work referred to above, which is interesting because it also seems to be the first attempt to use electrophoretic methods for the study of enzymic homogeneity and for purification of enzymes (ptyalin and pepsin). Ringer worked with pepsin prepared, according to Pekelharing, from dog's gastric juice, by dialysis and by precipitation with ammonium sulphate. To explain why the pepsin showed no isoelectric point, but yet had a marked pH-solubility minimum, Ringer assumed that the enzyme

was partially combined with protein, which would show positive charge at acid reactions. Electrophoresis in 0·025 N HCl gave, in fact, an anodic portion of high activity, whereas the cathodic portion was inactive but nevertheless contained much protein. At pH 4·1 no such separation was observed. As regards the dependence of the separation on pH Ringer's results are therefore contrary to Hammarsten's and Ågren's.

The following is an account of some experiments on the electrophoretic behaviour of solutions of crystalline pepsin. The apparatus and procedure earlier described by one of us [Tiselius, 1937, 1] were used. This method has several advantages, e.g. diminished risk of heat convection, possibility of simultaneous analytical and optical observation of the migration, and of more effective separation by applying higher voltage and the "compensation" device for balancing the migration.

A number of different crystalline pepsin preparations were investigated, which will be designated below by I, II, IIIa and IIIb. Pepsin I had been prepared according to Philpot's modification of Northrop's procedure [Philpot, 1935; Philpot & Small, 1938] and formed beautiful crystals about 0·5 mm. in length. Pepsin II was a glycerol solution of crystals (twice crystallized Cudahy Pepsin 12-7-3) prepared in Dr Northrop's laboratory, according to the procedure developed there. Philpot & Small (unpublished) have found that pepsin can be fractionated by salt precipitation to give an easily soluble crystalline fraction, and a less soluble non-crystalline fraction with a solubility minimum at pH 3·8, the latter having a somewhat lower specific activity (private communication of the authors). Samples of these two preparations dissolved in acetate buffer were kindly provided by Dr Philpot, and will be designated below as IIIa and IIIb respectively. The authors wish to express their thanks to Drs Northrop, Philpot and Small for generously placing material and information at their disposal.

The crystals were dissolved in the buffer solution to be used; the other preparations were first dialysed to remove glycerol. Before filling the apparatus all samples were dialysed overnight against the buffer to be used as a supernatant. Nitrogen was determined by the micro-Kjeldahl method, peptic activity by the haemoglobin method [Anson & Mirsky, 1932]. Like Philpot & Small [1938] we have found that the absolute values of specific activities by this method are somewhat dependent upon the preparation of haemoglobin used. Throughout this work, therefore, a single haemoglobin preparation was used.

Preliminary electrophoresis measurements showed that the various preparations contained one definite protein component of fairly homogeneous migration, with only small differences in migration velocity. Besides the sharp boundary given by this component, a very diffuse boundary could be observed, at least in concentrated solutions (more than 0·5%), indicating the presence of inhomogeneous material.

The final experiments involved optical determinations of the mobility of the main component at different pH (Table I, Fig. 1); in a number of experiments the optical observation was supplemented by analysis of the different compartments for nitrogen and peptic activity (Table II, Fig. 1), in order not only to correlate the activity migration with the migration of the main component but also to see how far the specific activity could be raised by electrophoretic separation. As the "analytical" experiments for this latter purpose were usually run for a much longer time, a higher buffer concentration (ionic strength 0·1) was used than in the "optical" series (ionic strength 0·02). In prolonged electrophoresis experiments disturbances due to electrolyte transport occur much more easily in dilute salt media than in more concentrated solutions.
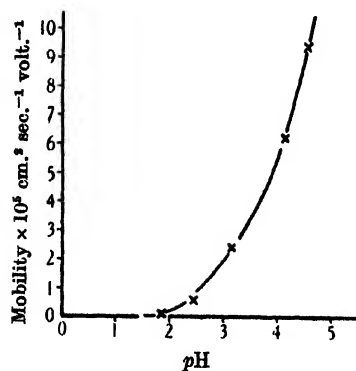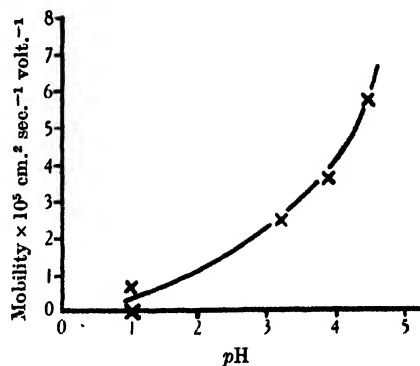
Fig. 1 a.   Fig. 1 b.

Fig. 1. Electrophoretic mobilities of the active component in pepsin
at $0°$ and ionic strengths (a) 0·02 and (b) 0·1.

Table I. *Electrophoretic migration velocity of main
component in solutions of crystalline pepsin*

Optical observation. Buffer ionic strength 0·02. Pepsin conc. 0·2–0·3%. Temp. 0°.

Preparation of pepsin	Buffer	pH	Mobility × 10⁵ cm.² volt⁻¹ sec.⁻¹
I	Acetate	4·57	− 9·4
IIIa, II	Acetate	4·15	− 6·2
I	Citrate, HCl	3·16	− 2·4
II	Citrate, HCl	2·47	− 0·6
II	Citrate, HCl	1·87	− 0·1

Determinations in 0·1 N HCl, pH 1·08 gave values for the mobility varying
between 0·0 and $−0·4 × 10^{-5}$. The variation is probably due to the fact that the
experimental error in this medium is much greater (too low buffering action).
No positive migration could be observed in any of the preparations.

Table II. *Electrophoretic migration and fractionation
of solutions of crystalline pepsin*

Optical and analytical methods used simultaneously. Ionic strength of buffer · 0·1. Temp. 0°.

Preparation of pepsin	Buffer	pH	Potential gradient volt/cm.	Time min.	Mobility × 10⁵ cm.² volt⁻¹ sec.⁻¹ Peptic activity	Total nitrogen	Main compartment (optical observation)	Specific activity $[P.U.]^{Hb}_{mg\ total\ N}$ Original solution	Top + compartment at end of exp.
I	Citrate HCl	3·19	7·64	195	− 2·68	− 1·38	− 2·42	0·162	0·273
I	HCl	1·08	3·06	315	±0·0	+ 0·6	±0·0	0·177	0·246
II	Acetate	4·41	7·65	595	—	—	− 5·65	0·214	0·344
II	Citrate HCl	3·19	9·30	515	—	—	− 2·40	0·213	0·324
IIIa	Citrate HCl	3·19	9·04	597	—	—	− 2·50	0·240	0·314
IIIb	Citrate HCl	3·19	9·39	493	—	—	− 2·32	0·175	0·250

The first two experiments in Table II were made with the purpose of measuring
migration velocities by the three different methods simultaneously, whereas
in the others we tried to obtain an appreciable separation by prolonged migration
(the main protein component migrated from 64 to 154 mm.) in combination
with compensation [Tiselius, 1937, 2]. Under these circumstances a change in the

composition of the bottom compartment was unavoidable and calculations of mobilities from analytical measurements in the usual way became impossible.

The results obtained in the experiments described in Tables I–II and Fig. 1 a, b may be summarized as follows. Solutions of crystalline pepsin, prepared by various methods, were in no case found to be quite homogeneous in electrophoresis, but contained some inactive nitrogen which could be removed by electrophoretic separation. In this way the specific activities of the various samples could be raised by 31–69 %. From the agreement between the mobilities (Table II, 6th and 8th columns) it is concluded that the peptic activity is associated with a protein component, which shows fairly homogeneous migration (does not give rise to more than one "schlieren" band in the optical observation arrangement). This protein in the pH range investigated (pH 1·08–4·57) carries a negative charge and shows no isoelectric point and thus behaves electrophoretically like an acid. Even a pepsin preparation which showed a solubility minimum at pH 3·8 (last experiment in Table II) behaved in principle in the same way.

Our experiments thus to a certain extent give further support to the view expressed by Northrop that the enzyme pepsin is a protein, but it is quite clear that pepsin crystals contain considerable amounts of inactive material, which may be removed by electrophoresis, and it seems likely that this material influences some of the properties of pepsin, such as solubility and acid-base combining capacity, in a marked way. The specific activity of crystalline pepsin according to Anson & Mirsky is 0·184 $[\text{P.U.}]^{\text{III}}_{\text{mg. protein N}}$, but in a recent publication by Herriot [1938] a higher value is given (0·26). We have preferred to refer our values to total nitrogen instead of protein nitrogen and (as seen from Table II) we obtained activities ranging from 0·162 to 0·240 for our starting material, but as high a value as 0·344 $[\text{P.U.}]^{\text{III}}_{\text{mg. total N}}$ for our most active fraction. In comparing these values with those obtained by other authors one must make the reservation that the haemoglobin preparations were not quite identical [see also Philpot & Small, 1938].

As regards the question of the homogeneity of pepsin it should be observed that the electrophoretic procedure is a very gentle separation method and could not break down a loose association complex, unless this were appreciably dissociated into its components. The homogeneity of the migration of the active component as found in the experiments described above is not therefore sufficient evidence per se for the chemical individuality of this enzyme, especially as the most active fractions obtained from different materials did not show the same specific activity (Table II). More experiments over a wider pH range and with systematically varied preparation methods are needed to clarify this question.

The somewhat surprising result that pepsin is not positively charged even in the most acid solutions investigated (pH 1·08) may possibly be due to an unusual tendency of the pepsin to combine with anions of the electrolyte medium. Such an effect has been previously found [Tiselius, 1930] for egg albumin, although not to as marked an extent as one would have to assume for pepsin to account for the above results. This would explain why, in the most acid solutions, the negative charge is larger when the ionic strength increases from 0·02 to 0·1 (see Fig. 1), contrary to the usually observed decrease in electrophoretic mobility with increasing salt concentration.

SUMMARY

Electrophoretic measurements show that the enzymic activity of solutions of crystalline pepsin, prepared by different methods, migrates as a homogeneous protein component with a negative charge and with no isoelectric point. Some inactive, inhomogeneous protein or protein breakdown material is left behind, and the specific activity may thus be raised by 31–69 %.

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CCXXXVII. THE FERMENTATION PROCESS IN TEA MANUFACTURE

I. THE ROLE OF PEROXIDASE

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THE fermentation of the tea leaf is a stage in the manufacturing process of tea which has received but little attention in the past. Various qualitative studies have been made at the several Tea Research Institutes but no thorough investigation has been made apart from that of Oparin *et al.* [1935].

Results in this field have a considerable commercial use and they are also of interest to the biochemist, not only as an illustration of the growing importance of enzyme chemistry in industry but also as a detailed study of the chemical changes taking place in damaged plant tissue.

Before dealing with the fermentation process itself it may be of advantage to outline the various stages in tea manufacture.

After plucking, the leaf is spread out in thin layers to undergo withering. During this process which usually takes about 18 hr. the leaf loses water and becomes flaccid. Unless this condition of flaccidity is produced the leaf is broken into flakes, instead of being twisted in the subsequent rolling process, and the resultant dry tea loses market value on account of its appearance. Not only is the withering process a physical preparation of the leaf for the further stages of manufacture but also some chemical changes may take place which partly determine the final quality of the manufactured tea. The nature of these changes however is still obscure.

The next stage in manufacture is rolling. The primary object of this process is to rupture the leaf-cells and to expose the expressed juices to the air. At the same time the tea receives its characteristic twist. As soon as the leaf tissue is in any way damaged fermentation starts, so that it is impossible to separate the rolling process from the next stage which is that of fermentation. After a period of about 1½ hr. rolling the leaf is spread thinly on a fermenting floor, usually of polished concrete, where the oxidation process which commenced in the rollers is continued for perhaps another 2 hr., after which the tea is fired to stop fermentation and to dry the leaf.

During fermentation oxidative changes take place which largely determine the flavour and colour of the infused liquor. The amount of tannin extractable by hot water is considerably reduced, and some of the colourless tannin is transformed into red and brown products which arise from the original tannin, partly by oxidation and possibly partly by condensation. The precise mechanism of these changes is not yet properly understood.

Theories of the fermentation reaction

Although the fermentation process has been ascribed to simple oxidation, and, at a later date, to the activity of enzymes of micro-organisms on the leaf, there has been very little doubt since the work of Mann [1901; 1903; 1904] that

the oxidation is a process catalysed by the peroxidases of the leaf itself. The fermentation process is but another example of the general post-mortem changes that set in after damage to plant-tissues.

Fermentation will not take place *in vacuo* but starts as soon as oxygen is admitted into the system. The oxidative process takes place only within certain limits of acidity, the pH optimum being about 5–6. Acidification stops fermentation, while addition of alkali accelerates a non-enzymic autoxidation of the tannin bodies. Small amounts of KCN completely inhibit fermentation, as does a preliminary steaming of the leaf. All these observations are characteristic of an enzymic oxidation, and proof of this is afforded by the isolation of a peroxidase from the leaf which rapidly browns a green leaf infusion in the presence of H_2O_2 as is described later in this communication. ·

While there can be very little doubt that the oxidative process is catalysed by enzymes of the leaf, there is no doubt that micro-organisms do exert a very considerable influence on the quality of the final product. As yet only a lowering of quality is recognized as a result of the uncontrolled development of micro-organisms during fermentation, but this does not exclude the possibility that there may be some beneficial results.

Nature of the enzyme and substrate in the tea leaf

Having established with reasonable certainty that the fermentation of tea is an oxidative process catalysed by enzymes, the first thing to establish is the nature of the enzyme involved.

Using a press juice from fresh green leaf, artificial substrates such as the "Nadi" reagent do give a positive reaction for oxidase, but the development of colour as seen in a toluene extract is very much intensified by the addition of H_2O_2. If the leaf is ground up with sand under alcohol the oxidase reaction becomes negligible while peroxidase activity is intensified. This intensification of peroxidase activity which is due to the removal of tannin will be discussed later. The disappearance of a weak oxidase reaction on washing with alcohol is probably due to removal of H_2O_2 formed in the leaf, small amounts of which are to be expected. Its presence would be revealed by a slight peroxidase reaction without the addition of further H_2O_2. As a result, therefore, of qualitative tests oxidase cannot be detected in the tea leaf, while peroxidase activity is very marked. This confirms the observations of Manskaya [1935] who ascribed the oxidative processes in the tea leaf to peroxidase action: oxidase could only be found in negligible quantities. Any mechanism for the fermentation process must take account of this quantity of peroxidase and also a reaction involving oxygen uptake not catalysed by polyphenol oxidase must take place, as fermenting leaf takes up a considerable amount of oxygen.

The work of Szent-Györgyi [1937] and Huszák [1937] makes it very probable that in peroxidase plants oxygen is transferred to metabolites by the following reactions.

$$\text{Ascorbic acid} + O_2 \xrightarrow{\text{ascorbic acid oxidase}} \text{dehydroascorbic acid} + H_2O_2$$

$$H_2O_2 + \text{catechol derivative} \xrightarrow{\text{peroxidase}} o\text{-quinone}$$

$$o\text{-Quinone} + \text{ascorbic acid} \longrightarrow \text{dehydroascorbic acid.}$$
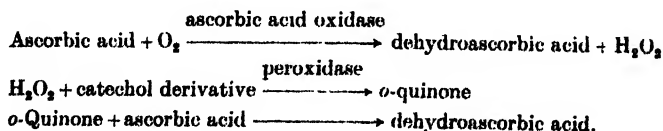
In normally respiring plant tissue the oxidation products are again reduced by activated H (dehydrogenase activity). It seems quite probable that the irreversible oxidations of damaged plant tissues are due to a dispersal of reducing

substances and coenzymes. Under these conditions the oxidation products formed in the above reactions find no activated H available and consequently undergo further transformations to form deeply coloured oxidation products of catechols.

It seems therefore very reasonable to suppose that in the fermentation of tea oxygen is absorbed, one of the products of oxidation being H_2O_2, and that the tea tannin is then oxidized by the H_2O_2 with the catalytic assistance of the peroxidase. This theory has been tested experimentally and found to be absolutely correct.

EXPERIMENTAL

If tea leaf is finely minced it undergoes the fermentation reaction very readily even when suspended in water. If such a suspension of minced tea leaf is kept *in vacuo* no browning of the tissues is apparent for several days, whereas on exposure to oxygen the leaf assumes a brownish tinge throughout in 2 to 3 hr. If dilute H_2O_2 (such that the final concentration of H_2O_2 does not exceed $N.50$) is added slowly to the minced leaf suspension, stored *in vacuo*, fermentation starts at once, and the suspension soon becomes uniformly brown. Anaerobic fermentation is therefore possible in the presence of H_2O_2. The same experiment shows that fermentation is probably catalysed by peroxidase.

It should be possible by mixing tea tannin with peroxidase and H_2O_2 to obtain the same chemical changes that occur in the actual fermentation process. Using a specimen of tea tannin kindly provided by Mr C. J. Harrison, and a tannin-free peroxidase extract from green tea leaf, the fermentation changes could not be simulated. Tannins, although tea tannin is not a true tannin, are known to have an inhibiting effect on peroxidase so that it was thought that the reason for this failure might be due to inactivation of the enzyme. Using a green leaf infusion instead of tea tannin, results indicating a satisfactory degree of oxidation were obtained. The infusion obviously contains substances which protect the enzyme from destruction by tea tannin.

On mixing the infusion, which, having been boiled, is free from enzymes, with a peroxidase solution, fermentation proceeds very slowly indeed, but on the addition of H_2O_2 the development of the brown fermentation colour is almost immediate. The optimum concentration of the H_2O_2 is about $N.200$. At higher concentrations the rate of fermentation is slowed down considerably and may even cease. To obtain the deepest brown colours it is best to add the H_2O_2 in small quantities at a time, never allowing it to accumulate in the system. This is in accordance with the well known susceptibility of peroxidase to H_2O_2 established by Willstätter & Weber [1926], Guthrie [1931], Mann [1931], and many others. H_2O_2 alone fails to oxidize tea tannin.

Tea tannin however only accounts for about 20% of the solid matter in a green leaf infusion, and the proper development of the fermentation colour in the experiment just described, while establishing the participation of peroxidase and H_2O_2 in the fermentation process, does not prove that it is the oxidation of the tannin to which this brown colour is due. Zender [1925] instances the oxidation of true tannins by oxidases so tea tannin is a likely substrate for peroxidase.

The same experiments were therefore carried out quantitatively, the tannin being titrated by $KMnO_4$ according to Löwenthal's method as modified by Carpenter & Harler [1922]. Incubation of a tea leaf infusion with peroxidase and H_2O_2, added in successive small quantities, materially decreased the tannin titre, any excess H_2O_2 remaining being decomposed by the catalase also present in the peroxidase solution. Oxidation appeared to come to a standstill after the

addition of a certain amount of H_2O_2 (Table I), but the amount oxidized was increased either by doubling the amount of enzyme or by adding further enzyme and H_2O_2 when the oxidation was apparently complete (Table II). The apparent completion of oxidation was therefore due to destruction of the enzyme in the course of the fermentation, a point of practical importance when considering the optimal time of fermentation in the manufacturing process.

It will be noted, from the results of Table II, that when peroxidase is in excess, the decrease in the tannin titre is greater than that which corresponds to the amount of H_2O_2 added. This has been observed on each occasion that excess peroxidase was present, and it would seem to indicate that the enzymic oxidation product of tannin can undergo further oxidation without the assistance of H_2O_2. It is well known that the oxidation of catechol derivatives rarely stops short at the first stage of oxidation, and subsequent stages of oxidation may take place readily without the aid of either enzyme or H_2O_2.

$KMnO_4$ titration is not specific for tea tannin in an infusion and it is usual to subtract from these figures the results obtained for non-tans. The tannin, in a larger aliquot portion than that used for crude tannin estimation, is precipitated with gelatin, precipitation being aided by salting out with acid and NaCl. The $KMnO_4$ titre of an aliquot part of the filtrate gives the non-tan value. Determinations of crude tannin and non-tan after incubation with peroxidase and H_2O_2 show that non-tans are also oxidized to much the same extent as tea tannin itself (Table III). These non-tans may of course be catechol bodies. In these experiments a filtered peroxidase solution was used, as it was found that the end point with indigocarmine was much sharper than with the unfiltered solution.

Table I

ml. $N/20$ H_2O_2 added	Crude tannin titration ml. $N/20$ $KMnO_4$	Difference
—	9·45	—
2·0	7·65	1·80
4·0	6·20	3·25
6·0	6·05	3·40
8·0	5·85	3·60

An infusion of 10 g. green leaf in 100 ml. of water and a peroxidase extract of 100 ml. from a further 10 g. of leaf were prepared according to the directions given later in this paper. 10 ml. portions of these solutions were mixed and 0·5 ml. portions of $N/20$ H_2O_2 added at intervals of 10 min. After addition of the H_2O_2 the solution was titrated with $N/20$ $KMnO_4$ in the presence of indigocarmine.

Table II

	Fall in tannin ml. $KMnO_4$
A. 5 ml. infusion + 5 ml. peroxidase + 2 ml. $N/20$ H_2O_2	2·0 ml. $N/20$ $KMnO_4$
B. 5 ml. ,, + 10 ml. ,, + 2 ml. ,,	4·2 ,,
C. 5 ml. ,, + two successive 5 ml. portions peroxidase and 2 ml. portions H_2O_2	5·0 ,,

Table III

	Tannin ml. $N/20$ $KMnO_4$	Non-tan ml. $N/20$ $KMnO_4$
Initially	7·5	4·55
At end of fermentation	4·8	2·1

5 ml. each of infusion and enzyme are mixed and four portions of 0·5 ml. $N/20$ H_2O_2 are added at 10 min. intervals. After the last addition another 5 ml. of enzyme and four more portions of H_2O_2 are added. For non-tan titrations five times these quantities are used.

In the actual fermentation process in manufacture both tannins and non-tans undergo oxidation but the oxidation of non-tan is relatively much less.

Table IV

	% Tannin	Non-tan*
Withered leaf	21·45	6·5 ml. $N/20$ KMnO$_4$
Leaf after 5 hr. rolling and fermentation	13·20	5·1 ,,

* As non-tans are ill-defined substances which have not as yet been separated they cannot be expressed on a percentage basis. They account for as much as 40% of the total permanganate titre of a tea extract. Shaw [1930] however is of the opinion that, in the gelatin precipitation, part of the tea-tannin molecule is split off and remains in the filtrate to be estimated as a non-tan.

The role of hydrogen peroxide

Reactions exactly analogous to those occurring in the fermentation process have been carried out *in vitro*, as just described, and the conclusion may be drawn that the browning of the tissue is due to peroxidase action on the tea tannin. A point unsettled in these experiments however is the participation of H_2O_2. This substance can be found only in very small quantities indeed in the fermenting leaf, but as it would be continually used up by peroxidase as soon as it was produced, and as the catalase present effectively checks its accumulation, direct methods of estimation would not be expected to reveal its presence.

In the past in discussing the role of peroxidase in plant respiration there has been considerable talk of the possibility of organic peroxides taking the place of H_2O_2 in peroxidase catalysed reactions. This is the theory adopted by the Russian workers in this field throughout the whole of their work.

There is no experimental evidence in favour of this view, in fact all the available evidence contradicts it. Wieland & Sutter [1930] found that mono-substitution of the H atoms in H_2O_2 by organic radicals materially reduced its ability to function as an oxidizing agent in the presence of peroxidase. Disubstituted products were completely without effect. Böeseken [1930] confirmed these results and added organic per-acids to the list of substances incapable of replacing H_2O_2. Szent-Györgyi [1925] has shown that *o*-quinones oxidize guaiacum without enzymic aid and we have shown that not only do *p*-quinones oxidize the Nadi reagent much more rapidly than the equivalent concentration of H_2O_2 in the presence of peroxidase, but that this oxidation by quinone is not accelerated by peroxidase. As there are several processes that can result in the formation of H_2O_2 in the leaf, and as the presence of catalase is a further indication of its presence, it will therefore be assumed that peroxidase oxidations in fermentation do actually utilize H_2O_2 and not some unspecified peroxide. The participation of H_2O_2 in the fermentation process is however capable of experimental verification. Catalase is a completely specific enzyme and if it could be shown that tannin oxidation is inhibited by catalase, it would prove that H_2O_2 is concerned in the fermentation process.

As shown before suspensions of minced leaf in water undergo fermentation. If excess of a liver catalase preparation is added to this suspension, fermentation is almost completely inhibited. If the catalase preparation is first inactivated by heat, fermentation proceeds unaffected.

It may therefore be concluded that H_2O_2 is concerned in the fermentation of tea, and as this fermentation is accompanied by a considerable uptake of O_2 it seems reasonable to ascribe the formation of H_2O_2 to this process. The nature of the O_2 uptake and the origin of the H_2O_2 will be investigated at a later date.

The oxidation of tannin is therefore a coupled oxidation such as that first established by Thurlow [1925]. Here secondary oxidation of guaiacum tincture by peroxidase was brought about in the presence of the H_2O_2 developed during the aerobic oxidation of hypoxanthine by xanthine oxidase. Kursanov's views [1935, 2], with the exception that he considers organic peroxides to be produced and not H_2O_2, are identical with ours. The absorption of oxygen and the production of H_2O_2 whether it is enzymic or not is preliminary to fermentation and does not lead to any development of colour.

Estimation of peroxidase activity in tea leaf

Having proved the importance of peroxidase in fermentation it becomes necessary to have some method of determining its activity in the tea leaf. The purpurogallin method, although the simplest, cannot be used in Assam throughout the whole year owing to the volatility of ether in the hot weather. The method of Guthrie [1931], with suitable modifications, proved to be the best available.

Technique and preparation of substrate and enzyme

The substrate was made up exactly according to Guthrie. 2·1 g. cryst. citric acid were dissolved in 17 ml. N NaOH and diluted to 100 ml. To 50 ml. of this solution 50 ml. of water were added 0·25 g. of p-phenylenediamine hydrochloride and 5 ml. of a 4 % solution of α-naphthol in 50 % alcohol. The solution was then filtered. 10 ml. of this reagent were then mixed with the enzyme solution (usually 2 ml.) and 2 ml. of $N/20$ H_2O_2 were then added. After 10 min. incubation the reaction was stopped by the addition of 2 ml. 1 % KCN. An aliquot portion, usually 3 ml., was taken and extracted with 25 ml. toluene and the depth of colour in the toluene layer estimated in a Lovibond tintometer. By a suitable mixture of reds and blues the colour could be matched exactly. A solution is said to have 6 Indophenol Units (I.U.) when the total reading of the red and blue slides is 6·0. The readings of the tintometer are not directly proportional to the concentration of indophenol blue, but a smooth calibration curve can easily be constructed. Determinations are accurate and reproducible to 0·1 I.U.

Tea juice or a suspension of the finely ground leaf in water cannot be used as the source of the enzyme as peroxidase activity is inhibited by tannin. Pretreatment of the leaf with alcohol seems to have no destructive effect on the enzyme, so the leaf sample is first ground up finely with sand under alcohol. The ground up tissue is filtered at the pump and the residue washed with alcohol until the washings are colourless. When all the chlorophyll has been removed the washings no longer show a positive tannin reaction.

After rapid drying *in vacuo*, to remove alcohol, the powdered residue is extracted with $M/15$ phosphate buffer (pH 7·0). A convenient enzyme solution is obtained by extracting the ground up residue from 10 g. leaf with 100 ml. phosphate. 1 ml. of the extract is then proportional to 0·1 g. of the original leaf. For determinations on single shoots (two leaves and a bud) the residue was extracted with 10 ml. phosphate, and an aliquot portion of this, 3–5 ml., was used in the estimation.

Testing of the method

The method described above must satisfy two conditions, the peroxidase must be extracted quantitatively from the leaf, and the amount of indophenol blue formed must be proportional to the enzyme concentration. That these conditions are fulfilled is shown in the following experiments.

Alcohol has very little effect on the activity of a peroxidase preparation even after 3 days' contact. Extraction of the peroxidase with the phosphate is quantitative, as if the tannin-free residue from 1 g. of leaf is extracted with 10 ml. of phosphate the activities of a 5 ml. portion of this extract and of the remaining 5 ml., together with the residue, are identical. Further, extractions for 5 and 15 min. gave enzyme solutions of identical activity.

The peroxidase activity of the final extract is much higher than that of an extract of fresh leaf owing to the tannin content of the latter. If to the final extract an amount of tea tannin equal to that originally present in the fresh leaf is added, it is found that the peroxidase activity of the extract is now depressed to the value it had originally in the fresh leaf. This has been established using both the indophenol blue and purpurogallin methods.
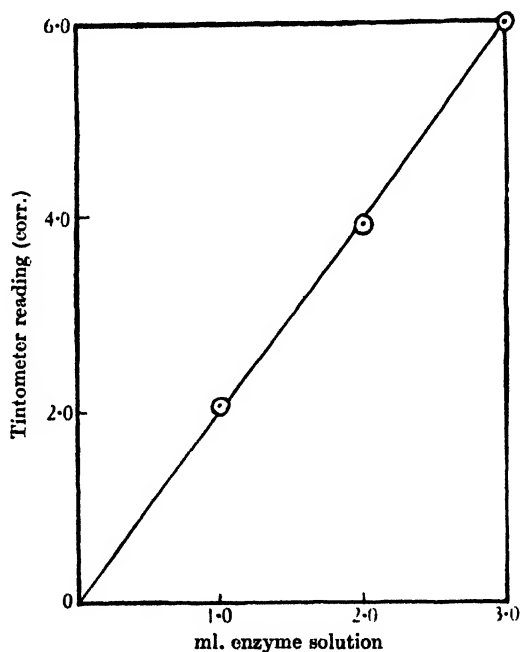
Fig. 1.

These experiments are considered to be sufficiently conclusive for it to be taken that extraction of the enzyme is quantitative.

The second requirement, that the extent of indophenol blue formation is proportional to enzyme concentration, is also fulfilled. The curve (Fig. 1) shows that the depth of colour is strictly proportional to enzyme concentration.

It is hoped to report the results obtained with this method in the study of fermentation at a later date.

Inhibition of peroxidase by tea tannin

Juice expressed from fresh tea leaf does not blue guaiacum even in the presence of H_2O_2. It was suspected that this was due to the inhibiting effects of tea tannin, and confirmation of this was found by showing that the addition of tea tannin inhibited the blueing of guaiacum by potato juice. If the tannin

is removed from the tea leaf by extraction with alcohol a positive peroxidase reaction is then given with guaiacum.

Tea tannin inhibits the formation of indophenol blue and of purpurogallin by peroxidase, but apparently to different extents. Table V gives the relative amounts of these two pigments formed in the presence of varying amounts of tea tannin.

Table V

	Indophenol blue	Purpurogallin
2 ml. enzyme	14·5	16·0
„ + 2 ml. 0·05 % tea tannin	7·6	14·1
„ + 2 ml. 0·5 % „	1·9	14·0
„ + 2 ml. 5 % „	0·5	9·5

The very marked inhibition of the indophenol blue reaction is partly apparent as tea tannin combines with indophenol blue and is not then extractable by toluene. If the tea tannin is added after the reaction has been stopped by KCN, most of the indophenol blue is fixed by the tannin and comparatively little is extracted by toluene. Acids have a similar effect so that the indophenol reaction must never be stopped by addition of acid as the weakly basic dyestuff is then retained in the aqueous phase on extraction with toluene. The inhibition of purpurogallin formation is a true enzymic inhibition; addition of tea tannin, after stopping the reaction, has no effect on the amount of purpurogallin formed.

In the above table, when 2 ml. 0·5 % tannin are added to the enzyme extract, tea tannin and peroxidase are in approximately the same proportions as they are in the leaf. The importance of removing tea tannin before estimating the peroxidase activity of a sample of leaf is at once apparent. True inhibition by tannin is not very high at this concentration but the degree of combination with indophenol blue is sufficient to make the results highly erroneous.

The theory has been advanced by Kursanov & Oparin [1929] that the inactivation of peroxidase by tea tannin can be minimized by protein decomposition products. In the leaf these were supposed by Kursanov [1935, 1] to originate by proteolysis during the withering stage of the manufacturing process. There seems no doubt that a green leaf infusion does contain protective substances as such an infusion is oxidized by peroxidase whilst tea tannin itself is not. On the other hand the evidence that these protective substances are protein decomposition products is slight.

To investigate this possibility experiments have been made using bacteriological peptone, which gives a precipitate with tea tannin, and a mixture of amino-acids. Neither had any effect over a whole range of concentrations on the formation of indophenol blue in the presence or absence of tannin. On the other hand both substances inhibited purpurogallin formation, presumably by combining with the quinone initially formed from pyrogallol, but had no effect on tannin inhibition. The effects of peptone (or amino-acid) and tannin were additive.

The suggested role of protein breakdown products in promoting fermentation receives therefore no confirmation.

Variation of peroxidase throughout manufacture

Some preliminary results are recorded here on the variation in peroxidase content of the leaf in the various stages of manufacture from green leaf to the final manufactured tea.

In each experiment 50 lb. of green leaf were manufactured. Samples were taken in duplicate of the green and withered leaf, at two different stages of rolling and at intervals throughout fermentation. When the fermented leaf was fired, part was retained to undergo further fermentation up to a period of 25 hr. The fired tea was also estimated for peroxidase activity. In cases where peroxidase activity was low, the amount of indophenol blue formed was increased either by increasing the amount of enzyme solution or by increasing the aliquot portion of the final incubation mixture. For fired tea both enzyme and aliquot had to be increased. Enzyme determinations were performed in duplicate. The sampling error was not great, only once did duplicates differ by more than 5 % and this was with leaf fermented for 25 hr. where bacterial development was very marked.

For the sake of economy in space the results obtained are not quoted in full. Enzyme activities are expressed in terms of I.U. per g. of dry tissue. The results obtained for a normal manufacture and for a protracted fermentation are tabulated in Tables VI and VII respectively.

Table VI

Green leaf	894	I.U. per g. dry wt. of tissue	
Withered leaf	1293	,,	,.
40 min. roll	780	,,	,,
90 ,,	685	,,	,,
2½ hr. fermentation	647	,,	,,
4 ,,	536	,,	,,
Fired tea	7	,,	,,

Table VII

Withered leaf	1051	I.U. per g. dry wt. of tissue	
90 min. roll	652	,,	,,
3¼ hr. fermentation	617	,,	.,,
5 ,,	178	,,	,,
6½ ,,	115	,,	,,
25 ,,	132	,,	,,

As has been noted before by Mann [1901; 1903; 1904] and Manskaya [1935] there is a definite increase in activity during withering. We also find a definite decrease in peroxidase activity during the first roll, after which the fall, although regular, is comparatively slight up to the point where fermentation is stopped by the firing process. If fermentation is prolonged beyond 4 hr. peroxidase activity again falls rapidly.

The earlier experiments described in this paper established the toxic effects of both tannin and H_2O_2 on the enzyme causing fermentation; in fact the enzyme could be completely inactivated. These factors are not so important in actual manufacture, as not only is H_2O_2 present in very low concentration but also resistance to tannin inactivation is much greater, owing presumably to similar protective bodies to those shown likely to be present in the green leaf infusion.

It is found that the firing process does not completely inactivate the peroxidase, 0·5 to 1·0 % of the original activity of the withered leaf being retained. Some of this activity is retained for at least 6 months. It is suggested that this residual activity may account in part for the post-fermentative changes which occur in the maturing of tea.

SUMMARY

A brief outline is given of the various stages in the manufacture of tea.

Oxidase is present in negligible quantities in the tea leaf and the fermentation process is brought about by peroxidase. The first stage in fermentation is an

uptake of oxygen leading to the oxidation of some as yet unidentified substance, and the production of H_2O_2. This oxidation is not necessarily an enzymic process and may be an autoxidation. The peroxidase then catalyses the oxidation of the tea tannin by H_2O_2 leading to the production of brown pigments which impart the characteristic colour to a tea infusion. Substances, grouped as non-tans, may also undergo oxidation.

No evidence is available for the utilization of organic peroxides by peroxidase, and in the fermentation of tea, decomposition of H_2O_2 by excess catalase inhibits tannin oxidation almost completely.

A quantitative method for the estimation of peroxidase in the tea leaf is described.

Peroxidase is inhibited by tea tannin although substances present in the leaf exert some protective influence against its effect. The theory that these protective substances are protein degradation products, formed during withering, finds no support.

An outline is given of the changes in peroxidase content of the leaf throughout the various stages in manufacture.

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CCXXXVIII. THE INFLUENCE OF THIOL GROUPS IN THE ACTIVITY OF DEHYDROGENASES. II

WITH AN ADDENDUM ON THE LOCATION OF DEHYDROGENASES IN MUSCLE

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IT was shown [Hopkins & Morgan, 1938] that when washed tissues are incubated anaerobically for adequate periods with solutions of glutathione (GSSG) at pH 7·4 the succinic dehydrogenase is completely inactivated. On the other hand the original activity of the enzyme is fully regained when the preparations so treated are subsequently re-incubated with the reduced form of glutathione (GSH). In the case of a second enzyme, the α-glycerophosphate dehydrogenase, the activity is wholly unaffected by the treatment described. Hopkins and Morgan suggested that these facts show that a thiol group in its structure is essential for the activity of the former enzyme, but not at all for that of the latter.

In experiments now to be described these observations have been confirmed and extended. A number of other dehydrogenases have been studied, but among them the succinic dehydrogenase was the only one found to respond to the influence of glutathione. The influence on dehydrogenases of other substances known or supposed to inhibit the action of hydrolytic enzymes by combining or reacting with SH groups in their structure has been investigated, and also the influence of these and other inhibitors in protecting the succinic dehydrogenase from the action of glutathione. Preliminary experiments will be described which seem to bear on the location of dehydrogenases in muscle fibres.

EXPERIMENTAL

Methods

In most of the experiments washed muscles from the hind legs of rabbits were employed, but in a few the enzyme preparations were made from other tissues. The muscles, immediately after their removal from the animal, were passed through a Latapie mincer and washed thrice with distilled water. Occasionally they were subsequently finely ground with sand in a mechanical mortar. The effect of various forms of treatment on the activity of the enzymes was in all cases determined by the anaerobic methylene blue technique; but in the cases of the succinic, glycerophosphate and lactic enzymes the degree of activity was also determined spectroscopically by observing the rate at which a preparation, in the presence of a substrate, reduced cytochrome c. In some experiments with succinic dehydrogenase the rate of O_2 uptake in the presence of cytochrome and substrate was measured.

(1829)

In the methylene blue experiments the procedure was made as nearly as possible the same in all experiments, so that usually in the individual protocols only the reduction times need be reported. In general 0·5 g. washed muscle was employed at this stage, or its calculated equivalent when, as a result of treatment, the tissue had gained or lost water. Small Thunberg tubes were used, and each tube contained, in addition to the tissue, 1 ml. phosphate buffer at pH 7·4 and 1 ml. water. To this was added 0·5 ml. $M/2$ substrate, the final concentration of the latter being therefore $M/12$. Incubation was always carried out *in vacuo* at 37°.

In all experiments which involved incubating the tissue with glutathione or other substances, the control sample used in the comparison of reduction times was itself incubated simultaneously in buffer solution without additions. The method of incubation was that described in the previous paper. The amount of methylene blue employed was uniformly 0·25 ml. of $M/1000$ solution.

I. The succinic dehydrogenase

That this enzyme can suffer complete loss of activity by adequate treatment with GSSG, and that its activity is restored on subsequent exposure to GSH solutions, has been repeatedly confirmed during our experiments. Since, as was pointed out in the previous paper, these reactions involve equilibria the complete removal of activity calls for more than one exposure to the GSSG solutions, though its complete restoration usually requires one exposure only to solutions of GSH of sufficient concentration. In many of the following experiments complete inactivation was not found necessary; wide changes in the degree of activity gave the information sought.

The suggestion has been made to us that the effect of GSSG might not involve an oxidation of SH groups as was assumed in the previous paper to occur, but might follow on the lines of some other inhibitions of enzyme activity. The constitution of the disulphide peptide is such that it might enter into a reversible association with the enzyme of a kind which would prevent essential groups in the structure of the latter from exerting their normal influence.

Such a view, unlikely from the general evidence discussed by Hopkins & Morgan [1938], seems to be disproved by experiments of the following simple kind. The process of inactivation, it will be shown, involves a reaction which has a prominent time factor and a somewhat high temperature coefficient, while its velocity increases continuously through a wide range of increase in the concentration of GSSG. In these respects it differs entirely of course from inhibition by agents which may be assumed to act by simply blocking the enzyme surface; e.g. malonic acid (see below).

Exp. 1. Latapie-minced and well-washed muscle tissue in portions of 0·5 g. was incubated anaerobically for 2 hr. at pH 7·4 with varying concentrations of GSSG. The samples were filtered off on a linen filter, well washed, and their reduction times determined as described above.

Exp. 1

	Reduction times
Original tissue	10 min.
Incubated with $M/100$ GSSG	17 min.
Incubated with $M/50$ GSSG	25 min.
Incubated with $M/10$ GSSG	2 hr. 30 min.

It is seen that the reduction time (inactivation of the enzyme) increases continuously with the concentration of GSSG.

Exp. 2 demonstrates the effect of incubation time on the degree of inactivation. Equal portions of washed muscle were incubated *in vacuo* for various times with 0·8 ml. *M*/5 GSSG, then filtered off and washed. One sample after incubation for 2 hr. was incubated for a second 2 hr. with a fresh supply of GSSG.

Exp. 2

	Reduction times
Original tissue	16 min.
Incubated for 25 min.	23 min.
Incubated for 60 min.	46 min.
Incubated for 2 hr.	1 hr. 30 min.
2 hr. sample re-incubated	∞

Exp. 3 shows the effect of temperature on the process of inactivation. Samples of muscle (0·5 g.) treated as before were incubated, with and without GSSG (*M*/10), for 4 hr. at different temperatures. Washed as usual.

Exp. 3

Temperature	Reduction times
20° control (no GSSG)	12 min.
with GSSG	15 min.
30° control	12 min.
GSSG	56 min.
40° control	12 min.
GSSG	2 hr. 40 min.

These three experiments show that inactivation by GSSG involves a progressive chemical reaction with a somewhat high temperature coefficient.

Cu as an inhibitor of the succinic dehydrogenase

It is well known that Cu reacts with SH groups and if the activity of the dehydrogenase calls for the integrity of such groups in its structure, it is to be expected that its presence will inhibit activity. This is the case. Quastel & Wooldridge [1927] found that treatment with $CuSO_4$ inhibited the succinic enzyme as well as some other dehydrogenases in the case of *B. coli* but we have found no reference to the effects of the metal on the enzyme of animal tissues.

Exp. 4. To equal samples of washed muscle, treated as usual and suspended in buffer solution, $CuSO_4$ was added to give the concentrations of Cu shown in the following table. The reduction times were determined immediately after the addition.

Exp. 4

Cu added µg.	Reduction times min.
0	10
5	11
10	12
40	15
100	80

It will be seen that with the amount of tissue used in this as in other experiments (0·5 g.) the metal has relatively little effect on the enzyme up to a concentration of 40 µg. in 3 ml. solution. With 100 µg. it becomes large.

These results bear on those of the earlier experiments of Hopkins and Morgan and on some of our own. By far the most convenient method for preparing GSSG from GSH is that of Pirie [1931] in which the latter is oxidized by H_2O_2 in the presence of small amounts of Cu. The oxidized product so made which has been chiefly used in this laboratory contains traces of Cu. This was ignored in the previous paper chiefly because it was found that cystine and cysteine absolutely free from Cu could in essentials replace glutathione in experiments described. It seemed to us desirable nevertheless to study the effect of these small amounts of the metal. The amount of Cu present in the preparations of GSSG was determined by the method of Callan & Henderson as developed by Haddock & Evers [1932] and found to be $10\,\mu$g. in 100 mg. (1 in 10,000). The presence of this amount as shown by experiments described below is not without effect on the apparent rate of enzyme inactivation by GSSG. It is sure however that the action of glutathione and that of the metal are distinct in kind. We have found that inactivation of the enzyme by GSSG occurs typically in the complete absence of Cu (Exp. 8).

Exp. 5 again illustrates the effect on the enzyme activity of small amounts $(10\,\mu$g.$)$ and of larger amounts $(100\,\mu$g.$)$ of Cu, and shows that in the case of the smaller amounts incubation does not increase the effect of the metal. This is exerted immediately. Of the tissue 0·5 g. was in 3 ml. buffer solution as usual. With each concentration of metal the reduction time was determined on one sample immediately after adding the metal. Two other samples in each case were incubated in its presence for two successive periods of 3 hr., the tissue being washed in between. One of the latter samples was then incubated with $M/10$ GSH solution. All reductions with succinate as substrate.

Exp. 5	Reduction times
No Cu	
Control untreated	12 min.
10 μg. Cu	
Determined immediately	14 min.
Incubated	13 min.
Incubated, then GSH	12 min.
100 μg. Cu	
Determined immediately	45 min.
Incubated	5 hr.
Incubated, then GSH	17 min.

The effect of $10\,\mu$g. Cu, the amount contained in 0·1 g. of the GSSG preparation described above (and therefore the amount usually present in our experiments with that preparation), though small, has been always observed and was of the same order in other experiments. It is noteworthy that with ten times this amount of metal, though marked diminution of the enzyme activity occurs at once, the effect increases with time. This circumstance will receive later reference. Subsequent exposure to the influence of GSH always restores the activity of the Cu-treated enzyme though usually not quite completely.

Experiments were carried out to determine to what extent the small amount of Cu in GSSG preparations prepared as described above is concerned in the inactivation observed when enzyme preparations are exposed to them.

Exp. 6. Dithiodiethyl carbamate is well known to form a complex with Cu. The following experiment was made to show (1) whether the carbamate has by itself any effect on the enzyme, (2) how far it prevents the effect of added Cu on its activity and (3) to what extent its presence affects the rate at which the activity

disappears when enzyme preparations are exposed to solutions of GSSG. 1 g. Latapie-minced washed muscle was incubated for 3 hr. in solutions at pH 7·4 containing the various additions mentioned. 2 ml. 0·1% carbamate were employed and in each case the total volume was 5 ml. After incubation the tissue samples were filtered off, washed and the reduction times determined as usual.

Exp. 6

	Reduction times
Control (muscle alone)	11 min.
With carbamate only	11 min.
With 10 μg. Cu	14½ min.
+ carbamate	11 min.
With GSSG (0·1 g. = 10 μg. Cu)	5 hr.
+ carbamate	3 hr. 50 min.

It will be seen that the carbamate had by itself no influence on the enzyme, while it protected it from the inhibitory effect of the added Cu. Its presence reduced the reduction time of tissue incubated with GSSG from 300 min. to 220 min. The effect of the metal seems to be in some sense additive.

It was felt desirable to add to the evidence provided by carbamate inhibition by using, if possible, GSSG preparations wholly free from Cu. We have found that if preparations containing such amounts of the metal as those made by Pirie's method are shaken in solution with an adequate amount of freshly washed minced muscle, the Cu is completely removed by adsorption on the tissue, or reduced to amounts too small to be demonstrated.

Exp. 7 was carried out with GSSG thus freed from Cu, and its effect on the enzyme compared with that of an equal concentration of the original preparation.

Exp. 7

	Reduction times
Muscle incubated without GSSG	5 min. 20 sec.
Incubated with the original preparation of GSSG	72 min.
Incubated with Cu-free GSSG	56 min.

The reduction time is again larger in the presence of the Cu.

In Exps. 6 and 7 the tissue was exposed only once to the GSSG solution. This, as has been frequently pointed out, is never adequate, at least with reasonable concentrations, to remove the enzyme activity completely.[1]

We wished therefore to show whether with adequate exposure the activity could be completely removed by a Cu-free preparation. This was the case, or nearly the case, in Exp. 8. The copper was removed by shaking 30 ml. $N/10$ GSSG at pH 7·4 with 2·25 g. of well-washed muscle. The tissue was filtered off after 5 min. Two samples of 1 g. tissue, prepared as usual, were incubated for two successive periods of 3 hr. each with 5 ml. $M/10$ Cu-free GSSG. A third equal sample was similarly treated but with the original GSSG preparation, and a fourth in the presence of 10 μg. Cu alone. All samples were prepared for the reduction time determinations in the customary way.

The Cu-free preparation reduced somewhat more rapidly than the original, but the difference was slight. The experiment showed clearly that contact with

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[1] The plan and extent of our experiments have been sometimes limited by the available supply of glutathione. Over 50 g. have been used in this research. All has been prepared in this laboratory.

GSSG when free from the metal can completely remove the activity of the dehydrogenase.

<div align="center">

Exp. 8

</div>

	Reduction times
Control (muscle incubated alone)	10 min.
Incubated with Cu-free GSSG	8 hr. +
+ carbamate	8 hr. +
Incubated with original GSSG	8 hr. + +
Incubated with 10 μg. Cu	14 min.

<div align="center">

Other inhibitors influencing SH groups

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Morgan & Friedmann [1938, 1] having shown that maleic acid by reacting with SH groups forms stable addition compounds with thiolacetic acid, cysteine and glutathione, showed further [1938, 2] that it reacts with the fixed SH groups of proteins and therefore inhibits such enzymic activities as call for the integrity of these groups. Hopkins & Morgan [1938] having shown that the activity of the succinic dehydrogenase comes into this category, the first-mentioned investigators were led to study the effect of maleic acid upon it, finding that well-marked inhibition occurs [Morgan & Friedmann, 1938, 2]. They had previously found however that interactions of thiols with the acid reach equilibria and do not proceed to completion. The following experiments confirm and extend their observations in the case of the succinic enzyme.

Exp. 9 involved a comparison between maleic and fumaric acids. Of a washed muscle preparation 3 equal portions were incubated overnight, alone in phosphate buffer (control), in $M/10$ maleic acid and in $M/10$ fumaric acid respectively, all at pH 7·4. Each portion was then submitted to the same treatment for a second time. They were then well washed at the centrifuge and their reduction times in the presence of succinate determined.

<div align="center">

Exp. 9

</div>

	Reduction times (min.)	
	Once incubated	Twice incubated
Control	12	12
Maleic acid	27	53
Fumaric acid	13	12

It is seen that the inhibition by maleic acid is marked but involves a slow reaction not going to completion. Fumaric acid has no effect upon the enzyme.

Rapkine [1933] has shown that iodoacetic acid reacts with the SH group of proteins, and its influence as an inhibitor of the dehydrogenase was discussed by Hopkins & Morgan [1938]. We have confirmed their results in finding that its action on the enzyme is a relatively slow process, increasing at any given concentration with the time of incubation. We have ourselves found that its effects are irreversible, not removed by washing the enzyme preparation or under the influence of GSH. In one experiment for instance involving the customary technique we found that an untreated preparation with a reduction time of 12 min. gave, after incubation with $M/25$ iodoacetic acid for 1 hr., one of 120 min.; and when afterwards incubated with GSH, 130 min. In another experiment, in which the control time was 12½ min., the preparation after exposure to iodoacetic acid required 91 min. although it was first very thoroughly washed. The effects are strictly irreversible.

Protection of the dehydrogenase from the influence of GSSG

Malonic acid is well known as an inhibitor of the activity of succinic dehydrogenase and must therefore enter into some effective association with its active structure. We therefore investigated its ability to protect the enzyme from oxidation by GSSG. In experiments for this purpose it is important to know that malonate can be easily removed from its association with the enzyme by washing the tissue preparations which have been exposed to solutions containing it. Unless the concentrations are high this is the case after relatively few washings as shown by the following experiments. Two 0·5 g. portions of a muscle preparation were placed in 2 ml. $M/10$ malonic acid. They were incubated anaerobically for $\frac{1}{2}$ hr., filtered off, and, in one case, the tissue was washed thrice on a linen filter before the reduction times were determined and in the other left unwashed. In the latter case the presence of the malonic acid on the surface naturally inhibited the enzyme. The control was an equal portion of the tissue incubated in buffer solution alone. The following reduction times were obtained. Control, 15 min.; treated with malonic acid and not washed, 61 min.; washed sample, 17 min. It is seen that the washed sample was nearly as active as the original. Although in some experiments we have first incubated the enzyme preparation with the malonic acid its association with the enzyme is in fact established immediately (Exp. 11).

The following experiments show that malonic acid can very completely protect the enzyme from oxidation by GSSG.

Exp. 10 shows that protection occurs with very low concentrations. Latapieminced washed muscle in portions of 0·5 g. was incubated for 2 hr. under the usual conditions with $M/10$ GSSG, respectively unprotected and protected by the presence of malonic acid in various concentrations. The following reduction times were obtained.

Exp. 10

	Reduction times
Original tissue untreated	9 min.
Incubated with GSSG unprotected	2 hr. 30 min.
with malonate $M/10$	15 min.
,, $M/50$	10 min.
,, $M/100$	11 min.
,, $M/500$	11 min.
,, $M/1000$	13 min.
,, $M/5000$	17 min.

It is clear that a large degree of protection is afforded by very low concentrations of malonate and that the effect decreases only slowly with a fall in these. It is probable that in the above experiment the tissue from the strongest ($M/10$) solution was not quite adequately washed and the small amount of malonic acid still adhering somewhat increased the reduction time.

Exp. 11 carried out with the same tissue preparation as in Exp. 10 shows that the formation of the association between malonic acid and the enzyme is a rapid process scarcely affected by a time factor. The tissue in $M/50$ malonic acid was incubated in Thunberg tubes with hollow turn-over stoppers containing $M/10$ GSSG. The latter was emptied into the tubes at successive intervals during incubation which in each case was afterwards continued for 2 hr. Tissue samples were filtered off and washed free from malonic acid before the reduction experiments were carried out.

Exp. 11

GSSG solution added	Reduction times	
	min.	sec.
At once	13	30
After 5 min.	12	30
After 10 min.	14	45
After 30 min.	14	40

Previous incubation is seen to have but little effect on the degree of protection from the influence of the GSSG. As shown in the protocol of Exp. 10 the reduction time of this preparation after exposure to the GSSG solution alone for 2 hr. unprotected was 2 hr. 30 min.

The following experiments illustrate further the efficiency of malonic acid in protecting the enzyme from the influence of GSSG.

Exp. 12. In this equal samples of a muscle preparation were incubated for two successive periods of 3 hr. each respectively in $M/10$ GSSG alone and in the same with $M/10$ malonic acid present. A control sample was, as in all experiments, incubated for similar periods in the buffer solution alone. After washing the following reduction times were obtained:

Exp. 12

	Reduction times
Control	5 min. 45 sec.
Incubated with GSSG alone	5 hr. +
In presence of malonate	8 min.

Exp. 13. In this the muscle tissue used was passed twice through the Latapie mincer with the intention of increasing its permeability. The experiment was otherwise essentially the same in plan as the foregoing but, in the incubations, while the concentration of GSSG was the same, that of the malonic acid was only $M/50$. The incubations were two, of 4 hr. each. The tissues were very thoroughly washed before the reduction times were compared.

Exp. 13

	Reduction times
Control	8 min.
Incubated with GSSG alone	5 hr. 30 min.
In presence of malonate	9 min. 30 sec.

Of *Exp. 13a* it need only be said that the procedure was similar, and the muscle preparation the same as in Exp. 13, but the incubation times were shorter and the enzyme therefore not completely inactivated by GSSG. The reduction times were as follow: control, 9 min.; after GSSG alone, 4 hr.; after GSSG plus malonate, 9 min.

The influence of substances, other than malonic acid, capable of protecting the succinic enzyme from the influence of GSSG was studied. Other dibasic acids displayed this property more definitely than other substances tried, among them, as might be expected, succinic acid itself, but also fumaric and malic; pyrophosphoric protects though generally less efficiently than the others.

The results of *Exp. 14* demonstrate such effects. A muscle preparation was incubated for 4 hr. with GSSG solution alone, and also in the presence of the substances shown. All samples were thoroughly washed.

Exp. 14

	Reduction times
GSSG alone	3 hr. +
With succinate $M/10$	4 min.
With fumarate $M/10$	3 min.
With malonate $M/10$	4 min.
With pyrophosphate $M/50$	4 min.

In this experiment, in which the enzyme preparation was exceptionally active, protection by all four substances was very complete. Other experiments have confirmed these results though, as stated, pyrophosphate has usually proved somewhat less efficient than the others.

It seemed worth while to try the effect of a greater variety of possible protectors. In the following (Exp. 15) the GSSG and the other substances were all employed in $M/10$ solution. The incubation period was 4 hr.

Exp. 15

	Reduction times
Original tissue (untreated)	10 min.
GSSG alone	3 hr.
With addition of:	
Acetate	4 hr. +
Phenylacetate	4 hr. +
Malonate	13 min.
Hydroxymalonate	20 min.
Lactate	2 hr.
Glucose	1 hr.
Sucrose	2 hr.

The monobasic acids other than lactic afford no protection. The reason for the definite but relatively smaller protection by lactic acid and glucose lacks explanation. Hydroxymalonic is nearly as efficient as malonic acid itself. Except for these two, none of the substances tried is an inhibitor of the enzymic activity.

Various authors have shown that the most efficient inhibitors of the succinic dehydrogenase activity are dicarboxylic acids [Quastel & Wooldridge, 1928; Potter & Elvehjem, 1937; Leloir & Dixon, 1937]. Two acidic groups seem to enter into special relations with the enzyme. It was to be expected that inhibitors will also protect the enzyme from oxidation.

Protection from the influence of GSH

It was desirable to discover whether substances which protect from oxidation by GSSG also protect from subsequent reduction by GSH. In the following experiment a muscle preparation was first incubated twice with GSSG, and the succinic enzyme present proved to be completely inactivated. The tissue was then divided into portions of 0·6 g. each, and these were incubated, one sample with GSH alone, and others with the substances indicated below. The GSH was in $M/10$ solution, buffered as usual. It was placed in the hollow stoppers of Thunberg tubes and added after the samples of oxidized tissue had been incubated anaerobically for $\frac{1}{2}$ hr. with the materials under study. The samples were then washed and their reduction times determined.

Succinic and fumaric acids inhibit the reduction to some extent; the others not at all. That malonic acid protects from GSSG so completely and from GSH not at all suggests structural relations which may prove instructive.

Exp. 16

	Reduction times min.
Control (tissue untreated)	12
Oxidized by GSSG	∞
Re-reduced by GSH alone	12
GSH + succinic acid	19¼
+ malonic acid	13
+ fumaric acid	17
+ pyrophosphoric acid	12½

Protection of the enzyme thiol groups from the influence of GSSG

When the nitroprusside test is used to compare the concentration of SH groups in active and inactivated preparations respectively it should be realized that a very low intensity in the test is compatible with activity.

If SH groups are necessary for the activity of the succinic dehydrogenase, and for this there seems to be strong and cumulative evidence, it is clear that malonic acid and other protectors of the enzyme activity must prevent that group from being oxidized by GSSG. It is by no means easy to prove directly that this is the case, nor is it perhaps easy to picture a mechanism for such protection. Malonic acid, for instance, apparently establishes no special relations with thiol groups, so that the protection must in any case be indirect.

So long as we remain unable to isolate the enzyme, or obtain preparations in which it is present in higher relative concentrations than in whole tissues merely washed, it is somewhat difficult to employ the nitroprusside reaction in proof that the SH groups proper to the enzyme itself have or have not been protected. They must represent but a minute portion of the thiol groups present in the tissue as a whole. That the active surface of the specific enzyme compared with the surfaces presented by disintegrated muscle must be exceedingly small cannot be doubted, and this seems to be illustrated by the minute concentration in which malonic acid can protect the enzyme (Exp. 10).

If the nitroprusside reaction given by a tissue sample which has been exposed unprotected to GSSG be compared with that given by a corresponding sample protected by malonic acid, though the activity of the enzyme is nearly lost in the former and intact in the latter, the reaction may be slight in both cases and a difficulty may be felt in deciding whether there is a significant difference between them. It is however easier to distinguish if the two samples be extracted with trichloroacetic acid and the test applied to the solution. It can be said definitely that in our experiments, though the difference may have been slight in some cases, it has always been more intense in a protected sample. It seemed desirable however to obtain some degree of quantitative evidence for this, and we have succeeded in doing so by incubating anaerobically with solutions of GSSG the samples to be compared. These were then titrated with very weak standard I_2 in the presence of KI in order to determine the amount of GSH produced by the interaction between the protein SH groups in the tissue preparation and the GSSG in solution. The quantities involved are doubtless too small for accuracy, but they allow of comparisons which are significant.

In each of two experiments sufficient of a preparation of muscle was made to carry out the above treatment, as well as determinations of enzyme activity. It was incubated with GSSG anaerobically in large Thunberg tubes for adequate periods, with and without the presence of malonate. The respective reduction times showed, in each experiment, that in the absence of protection the enzyme activity had nearly disappeared, somewhat more completely in the first experi-

ment than in the second. It was fully preserved in the presence of malonate. After incubation as above the evacuated tubes were opened under a solution of trichloroacetic acid, the tissue was filtered off and the filtrates titrated with $N/5000$ I_2. The end points could be observed without difficulty. The following I_2 equivalents are calculated in each case for 5 g. tissue preparation.

	ml. $N/5000$ iodine	
	Exp. 1	Exp. 2
Incubated with GSSG alone	1·2	7·8
Incubated with malonate present	16·0	19·5

There seems to be no doubt that the enzyme SH groups are protected by malonic acid in some specific way.

We may add that in an occasional experiment when treatment of the tissue with GSSG had made the nitroprusside reaction completely negative the enzyme was found to be inactive. This result is relatively easy to obtain, as Hopkins & Morgan [1938] found, when heart muscle is the source of the enzyme. With the less permeable preparations from skeletal muscle the treatment must be prolonged and repeated. That the enzyme structure nevertheless remains intact save for the disappearance of the SH groups may be shown by subsequent treatment with GSH when the activity is fully restored.

Oxidation of SH groups by alloxan

It was shown by Purr [1935] that papain and cathepsin are inactivated by alloxan owing to the fact that it oxidizes SH groups in the structure of these enzymes. We have tested its effect on succinic dehydrogenase and find that in adequate concentration it inactivates it, the effect being reversible (though apparently not completely) under the influence of GSH. The presence of succinic acid protects from oxidation by alloxan, but malonic gives relatively very little protection. The following experiments illustrate these statements. It should be here remarked that the nitroprusside test shows that alloxan oxidizes the SH groups of a muscle preparation very quickly, even in low concentrations ($M/100$, $M/50$). When used in $M/10$ solution it gives the familiar pink colour with the muscle protein which has been recently studied by Lieben & Edel [1932].

In the following experiment a muscle preparation, made as usual, was incubated for 30 min. with $M/100$ and $M/50$ alloxan. Samples were well washed and their reduction times determined. The activity of the α-glycerophosphate enzyme was determined at the same time, so it is convenient to give the results here. Other samples of the oxidized material were washed and then incubated for $1\frac{1}{2}$ hr. with $M/10$ GSH, again washed thoroughly and submitted to reduction tests. In the protocol SD = the succinic and GLD = the glycerophosphoric enzyme.

Exp. 17

		Reduction times min.
Control untreated	SD	15
	GLD	8
Alloxan $M/50$	SD	50
	GLD	8
Alloxan $M/100$	SD	56
	GLD	8
GSH after alloxan $M/50$	SD	20
	GLD	8
GSH after alloxan $M/100$	SD	22
	GLD	8

The glycerophosphoric enzyme is seen to be unaffected by alloxan at this concentration. GSH reverses its effect upon the succinic enzyme. Other experiments have given similar results. The following shows that the alloxan reaction is progressive with time. The preliminary treatment of the samples was the same as in the last case.

Exp. 17a

	Control	$M/50$ alloxan filtered off immediately	$M/50$ alloxan incubated 30 min.
Reduction times (min.)	SD 11	15	60
	GLD $4\frac{1}{2}$	$4\frac{1}{2}$	$4\frac{1}{2}$

Finally the following shows that as stated above the protection of the enzyme by succinic and malonic acids from oxidation by alloxan differs in an interesting way from the protection they afford from oxidation by GSSG. Succinic acid affords a considerable degree of protection, malonic very little.

	Reduction times (min.)
Control	10
Alloxan alone	35
Alloxan + malonate	26
Alloxan + succinate	14

Alloxan in concentrations of the order of $M/10$ has effects on the enzyme which must be additional to the oxidation of thiol groups, and when incubated with it all the dehydrogenases we have studied lose much of their activity.

II. OTHER DEHYDROGENASES

We have conducted experiments, precisely similar in kind to those of the previous section, with the α-glycerophosphate, lactic and malic dehydrogenases; also with the aldehyde mutase and alcohol dehydrogenase of horse liver (prepared by the method of Dixon & Lutwak-Mann [1937]) and with two enzymes of the oxidase type, namely the cytochrome (indophenol) oxidase and the xanthine oxidase. None of these enzymes is appreciably influenced by glutathione. Consistent with this is the circumstance that their activity, except for that of the liver aldehyde mutase, is not affected, or affected to a small extent only, by inhibitors which are believed to act by suppressing the influence of thiol groups.

The experiments with the succinic dehydrogenase have been described in detail and those of the present section were essentially similar in all respects. The evidence can therefore be more briefly presented. To the lactic and malic enzymes cozymase was of course added when the reduction times were determined.

Effects of incubation with GSSG and GSH

In the following table the results of several experiments are collected.

	Reduction times (min.)			
Enzyme	Original	GSSG alone	GSSG with malonate	GSH
Glycerophosphate	6	$6\frac{1}{2}$	6	6
,,	10	10	10	10
Lactic	3	5	—	—
,,	$3\frac{1}{2}$	5	$4\frac{1}{2}$	$5\frac{1}{2}$
,,	$4\frac{1}{2}$	7	7	—
Lactic (from horse liver)	20	21	—	22
Malic	$6\frac{1}{2}$	$8\frac{1}{2}$	—	—
,,	9	13	—	—

In all cases the influence of GSSG was absent or negligible.

Effect of Cu

The relatively small effect of Cu on the lactic dehydrogenase is illustrated in the following experiment. The preparation was not incubated with the metal but the effect was tested immediately after its addition.

	Reduction times min.
Control (+ cozymase and lactate)	3¼
With 5 µg. Cu	4
With 50 µg. Cu	6
With 5 µg. Cu (+ dithiodiethylcarbamate)	3½
With 50 µg. Cu (+ dithiodiethylcarbamate)	3¾

In the cases of the lactic and malic enzymes the differences between the effect of incubation with the GSSG preparation containing 10µg. Cu in 100 mg. of GSSG (see previous section) and that with the Cu-free preparation were compared in exactly similar circumstances. The results should be compared with Exp. 7 in which the same tissue was employed.

	Reduction time (min.)	
	With lactate	With malate
Tissue incubated alone	2½	6
With GSSG containing Cu	5½	8
With GSSG Cu-free	2¾	7¼

In one experiment with the glycerophosphate enzyme 10µg. Cu were added to two equal portions of a tissue preparation, treated otherwise as usual. In one case the reduction time was taken immediately; in the other the tissue was first incubated with the metal for 3 hr. The control was reduced in 7 min.; the sample with Cu, unincubated, 8 min.; the incubated sample 10 min.

Effects of maleic and iodoacetic acids

The following experiments illustrate the absence of appreciable inhibition due to maleic (and fumaric) acids. The results were confirmed in other cases.

	Reduction times (min.)		
Enzyme	Controls	Maleic acid	Fumaric acid
Lactic	3	3¼	3
Glycerophosphate	4	4	4
Malic	14¼	15¼	14¼

The following show that the same is true of iodoacetic acid even after incubation with $M/50$ solution for 1 hr.

	Reduction times (min.)	
Enzyme	Control	Incubated with I.A.
Lactic	3½	4
"	3¾	3½
Glycerophosphate	7	10

In this group of dehydrogenases, as will be seen from the various protocols given in this section, the influence of glutathione is absent, or, in comparison with the case of the succinic enzyme, negligible. The glycerophosphate enzyme has always shown remarkable resistance to the effects of the treatment involved in our experiments, its reduction times remaining constant at all stages; the lactic and malic enzymes have proved somewhat less stable but it will be seen

that their resistance to glutathione is definite enough. Consistent with this, in proof that thiol groups are not essential to their activity, is the circumstance that these enzymes are much less affected by Cu than is the succinic enzyme, and, unlike it, are not affected by the irreversible effects of maleic and iodoacetic acids.

The influence of glutathione on two other enzymes was tested, namely the aldehyde mutase and the alcohol dehydrogenase from horse liver. In neither case did exposure to GSSG affect the activity though the mutase has been said to be sensitive to iodoacetic acid [Dixon & Lutwak-Mann, 1937].

Oxidases

Experiments made with the Cu-containing preparation of GSSG described in the previous section led us at first to believe that the xanthine "oxidase" resembled the succinic enzyme in its relations with glutathione. This however proved to be due to its great sensitiveness to Cu, a property which had been previously recorded by others [cf. Andersson, 1936]. Small amounts of Cu equivalent to that contained in the glutathione removed the enzyme activity to an equal degree. The effect of Cu is however reversible and completely removed by treatment with GSH. It would seem that this enzyme may prove not to be a typical oxidase, but at the moment it may be left with the title.

The cytochrome (indophenol) oxidase was also studied. Its activity was measured by using phenylenediamine as a substrate and also by testing spectroscopically its ability to oxidize cytochrome. Its activity was found to be entirely resistant to the influence of GSSG.

CYTOCHROME-REDUCTION METHOD FOR DETERMINING ENZYME ACTIVITY

It is of interest in the case of those dehydrogenases which reduce the pigment to observe with a spectroscope the rate at which the prominent bands of reduced cytochrome c appear when the oxidized form is added to a solution containing

| Enzyme preparations treated with following substances | The spectrum of reduced cyctochrome c on adding the substrate became: | | | | | |
| | Just visible after | | | Fully developed after | | |
	SD	GLD	LD	SD	GLD	LD
Control untreated	At once	At once	At once	50 sec.	20 sec.	25 sec.
GSSG	No bands at any time	40 sec.	50 sec.	No bands	1 min.	3 min.
„ then GSH	At once	At once	1 min.	20 sec.	20 sec.	2 min.
„ Cu-free	No bands at any time	—	—	No bands	30 sec.	1 min.
„ +malonate	2 min.	—	—	4 min.	—	—
„ +succinate	5 min.	—	—	7 min.	—	—
„ +pyrophosphate	8 min.	—	—	13 min.	—	—
Cu high concentration	40 min.	—	5 min.	Several hours	—	10 min.
Cu low concentration	2 min.	1 min.	2 min.	4 min.	2 min.	5 min.
Alloxan $M/50$	60 min.	1 min.	—	2 hr.	3 min.	—
Iodoacetic acid $M/80$	22 min.	2 min.	2 min.	1 hr.	3 min.	15 min.
Iodoacetic* acid (muscle in vivo)	1 hr.	—	—	Several hours	—	—
Maleic acid	17 min.	—	—	30 min.	—	—

* In this experiment iodoacetic acid was injected into the lymph sac of frogs. One hind limb was removed before the injection as a control, and the motor nerves to the muscles of the other limb were cut. When rigor was evident in the forelimbs the second limb was removed. The muscles in each case were then ground up with a little water and washed till free from haemoglobin, and the succinic dehydrogenase activity determined spectroscopically. In the control the cytochrome bands were fully established in 30 min.

an enzyme and its specific substrate. It forms a check on the methylene blue results and is a convenient and rapid method for deciding whether an enzyme is present or absent, giving also approximate information as to the degree of its activity. It will be seen on examining the above table that results obtained with the methylene blue method are confirmed in every instance. The method of observation was of the simplest. A test tube containing the enzyme preparation, suspended in phosphate buffer at pH 7·4 together with a moderate concentration of oxidized cytochrome c, was clamped before the slit of a fixed straight vision spectroscope with an adequate light source.[1] A stopwatch was used and the times taken from the moment of adding the substrate (1) to the first observable appearance of the bands, and (2) to the establishment of their maximum intensity. The descriptions given in column 1 of the table will be understood from what has gone before.

The following is an experiment in which was measured the O_2 uptake by systems containing tissue preparations which had been previously treated with GSSG, unprotected and protected. One sample after oxidation was re-reduced with GSH. The substrate was succinate, and cytochrome c was added to the system. The necessary cytochrome oxidase was present in sufficient concentration in the muscle preparations. Barcroft manometers: O_2 uptakes are given in μl. per hr.: reduction times for the corresponding samples.

Previous treatment of tissue	O_2 uptake	Reduction times
Original	280	11 min.
Incubated with:		
GSSG alone	0	7 hr. +
,, with malonate	210	14 min.
,, with succinate	190	19 min.
,, with pyrophosphate	140	16 min.
,, then GSG	310	10 min.

It will be seen that as measures of enzyme activity the two methods are in close correspondence.

ADDENDUM

Location of the dedydrogenase in muscle

We have found that if muscle tissue minced with the Latapie is thoroughly extracted with salt solutions as for the extraction of myosin etc., the extraction being continued until no trace of protein is present in a final extract, the fragmented fibres, of which the majority when examined under the microscope are seen to be severed transversely, still show cross striation and—as we have repeatedly observed—typical birefringence of the discs. That cross striation remained intact after continued extraction with NH_4Cl was observed by Danelewsky [1882]. That author believed—perhaps on not very convincing evidence—that a relatively high proportion of this "framework" in their fibres was characteristic of muscles with the greatest functional activity. We have found that muscle tissue after exhaustive extraction with salts still contains succinic, α-glycerophosphoric, lactic and malic dehydrogenases in relatively high concentrations.

The saline extracts, unless they obviously contain dispersed colloidal particles, are found to display no dehydrogenase activity. The later extracts of a series of extractions can be obtained, after centrifuging, perfectly clear, and these are quite free from these enzymes though in the extracted residue their concentrations are high. If the completely extracted tissue be now treated with, say, 10 times its volume of $N/100$ HCl, more protein is extracted and the final residue

[1] The experiments with the lactic dehydrogenase were carried out anaerobically.

becomes gelatinous. It can however be centrifuged off in a high speed centrifuge and is then found to be inactive, as is also the protein extracted by the acid. The framework structure of the fibres immediately disappears on treatment with HCl.

It is true, to judge from methylene blue reductions, that the concentrations of the enzymes when compared with those in the original tissue are not increased proportionately with the removal of so large a proportion of the muscle substance (some 85 % of the total muscle proteins), so that what happens during the prolonged treatment is not entirely clear. The facts stated may be illustrated by the following experiment, typical of several made.

Latapie-minced muscle (25 g.) was first extracted four times with 200 ml. NaCl (10 %) and centrifuged after each extraction: the second extract stood overnight in the refrigerator. It was then extracted thrice with LiCl [Bate Smith, 1935; 1937]. The last extract contained no trace of protein. The final residue in which the fibres showed typical cross striation was washed and then centrifuged until freed as far as possible from water. Equal weights (0·5 g.) were used in determining the following reduction times, but the equivalence of this weight with that of the original minced muscle was not accurately known.

<div align="center">Reduction times</div>

Dehydrogenase	Control	Incubated GSSG	Re-incubated GSH
Succinic	12 min. 30 sec.	5 hr.	10 min.
α-Glycerophosphate	6 min.	6 min.	6 min.
Lactic	5 min. 30 sec.	11 min.	—
Malic	8 min. 40 sec.	13 min.	—

It will be seen that the enzymes in the extracted tissue show the same relations as those always observed with tissue when simply washed with water.

In a special experiment in every way similar to this the enzyme activity of the saline extracts was examined. The order of the extractions was the same as in the above. A sample of the fourth NaCl extract and one of the first LiCl extract were dialysed and in each case the precipitate which separated was centrifuged off, washed and tested for enzyme activity. The precipitate from the NaCl extract contained none of the succinic but very small amounts of the glycerophosphate enzyme and doubtful traces of the others. That from the LiCl extract was practically free from any activity. All the enzymes were present in the residue in concentrations comparable with those above. The supernatant solutions in the dialyser were free from activity.

It seems clear from the recent work of Weber [1934], Edsall & von Muralt [1930], Bate Smith [1935] and others that the protein which resists salt-extraction is mainly myosin itself, protected in some way from removal. In the case of Latapie-minced muscle it seems unlikely that this is due to the impermeability of the membranes, as the fibres are almost all broken across. Bate Smith [1937], who confirmed the presence in muscle of "globulin x" a protein first described by Meyer & Weber, is of opinion that some of this is associated with myosin in the residue after salt extraction (personal communication). It would be of interest, as he suggests, if, while myosin molecules form the basis of the contractile substances as is now generally admitted, the enzymes were associated with the above globulin which is in close relation with the myosin.

The circumstance that during and after the salt extraction the dehydrogenases seem to remain confined to the residual structural framework, while the degree of consequent increase in their concentration is not what would be

expected, calls for explanation. It is conceivable that denaturation changes during the extractions may be in part responsible for this.

In any case it is of interest to know that enzymes controlling energy-yielding reactions exist in this close relation with the structural framework of the fibres.

DISCUSSION

The experiments in this paper have fully confirmed those of Hopkins & Morgan in showing that the activity of the succinic dehydrogenase is completely removed when preparations containing it are incubated with GSSG and fully restored on subsequent incubation with GSH. Those authors showed that, in contrast, the α-glycerophosphate dehydrogenase is wholly unaffected by this treatment. We have extended the list of resistant enzymes by the inclusion of the lactic and malic dehydrogenases, the alcohol dehydrogenase and the aldehyde mutase from horse liver, xanthine oxidase and cytochrome oxidase. Rapkine [1938] has recently shown that the triosephosphate mutase from muscle may be inactivated by GSSG; but, to judge from our results, the succinic enzyme seems likely to prove the only typical dehydrogenase which undergoes this inactivation, and therefore probably the only one which requires for its activity the integrity of the SH groups in its specific protein structure. We have added to the evidence for the importance of such groups in the case of that enzyme by showing that Cu, maleic acid and iodoacetic acid, which are known or believed to react with thiol groups, are inhibitors of the enzyme, while having little or no effect on the activity of the other dehydrogenases tested. Of the above, Cu inhibits reversibly; the two acids irreversibly.

Nearly all our experiments have been made on skeletal muscle tissue, but Hopkins & Morgan showed that, so far at least as the effects of glutathione are concerned, preparations of the enzyme from cardiac muscle, kidney and liver behave identically. The evidence that these effects are due to oxidation and re-reduction of thiol groups is in any case strong, but we have perhaps added to it by showing that the inactivation by GSSG involves a progressive chemical reaction with a somewhat high temperature coefficient (Exps. 1, 2, and 3). If the inactivation were due merely to the formation of some reversible association of GSSG with the enzyme in which the latter is inactive but which dissociates under the influence of GSH (this being perhaps possible though unlikely) its kinetics would not be of this kind.

Alloxan, as an oxidant of SH groups, when in relatively low concentrations ($M/50$, $M/100$) inactivates the succinic dehydrogenase. In contrast, at such concentrations it has no effect at all on the α-glycerophosphate enzyme (Exp. 17). At high concentrations ($M/10$), when it produces the characteristic pink colour with the tissue proteins, it exercises a less specific destructive effect on all the enzymes we have tested. If the concentrations used are of the lower order its effects on the succinic enzyme are reversed by reduction with GSH; otherwise the reactivation is only partial.

Cu is well known to react with thiol groups and it was to be expected that an enzyme of which the activity calls for the integrity of such groups in its structure would be inhibited by the presence of the metal. It proved easy to show that this is true of the succinic dehydrogenase. With adequate Cu concentrations the inhibition is complete and immediate. With tissue preparations however, such as have been generally used by others and by ourselves in studies of this enzyme, certain difficulties arise in points of detail, and these were evident when the effects of varying the concentration of Cu were being studied.

Since Thunberg [1909] first demonstrated the existence of this dehydrogenase most experiments made with it, as well as those with other "insoluble" dehydrogenases, have involved the use of whole tissues which were merely minced (or sometimes more completely disintegrated) and then thoroughly extracted with water. In any such preparation the specific protein of any one enzyme is in association with those of other enzymes, and, of course, also with a relatively very large proportion of indifferent tissue proteins. In numerous experiments this circumstance has proved to be of no consequence, as the literature of the subject shows. When, however, the significance of some detail in the specific enzyme protein is to be determined difficulties may intrude. When for instance Cu is added to such a tissue preparation it must be expected to react with the SH groups of the indifferent tissue proteins present no less than with those proper to the enzyme, and, of course, the number of the former present must be relatively very great. Only a small fraction of the metal added may therefore be effective. An added amount which, were it possible to work with the enzyme in a concentrated form, might produce strong inhibition may with such tissue preparations have little effect. This was the case with the lower concentrations of Cu employed in our experiments (10–20 μg. per g. tissue). Inhibition even with these was always to be observed, but it was slight.

With adequate concentrations of Cu, which are relatively large (in the conditions of our experiments 300 μg. for each g. muscle tissue), inhibition, as already stated, is immediate and complete. With intermediate concentrations (e.g. 100 μg.) it might seem from Exp. 5 that a time factor is involved in the process. This however is unlikely and it would seem rather that the nature of the enzyme preparation is again involved, and that a permeability factor intervenes. While with such concentrations the amount of Cu reaching the dehydrogenase itself may be adequate to induce noteworthy inhibition, it yet takes an appreciable time after its addition to the washed tissue to reach the enzyme surface.

These various relations seem to call for comment, especially as they bear in a general way on the customary use of such tissue preparations. They do not however obscure the proof that the activity of the dehydrogenase can be completely inhibited by Cu. On the other hand our experiments have shown that the lactic, malic and α-glycerophosphate dehydrogenases, in keeping with their resistance to oxidation by GSSG, are affected to a very much smaller extent by the presence of the metal.

We had occasion in the experimental section of this paper to point out that the GSSG used by Hopkins & Morgan [1938] and by ourselves in most experiments, owing to the method of its preparation contained a trace of Cu. The relative slight effect of this on the rate with which such preparations inactivate the succinic enzyme is discussed in the earlier section. That inactivation proceeds typically under the influence of GSSG in the complete absence of Cu is shown in Exp. 8.

It should be noted that inhibition by Cu is reversible and is removed when the enzyme preparation is subsequently incubated with GSH (e.g. Exp. 5).

We have confirmed the results of Morgan & Friedmann who having found that maleic acid and SH-compounds interact to form stable addition compounds, showed that the acid also reacts with the SH groups of proteins and thus inhibits the activity of succinic dehydrogenase. This inhibition is not complete, as an equilibrium is earlier reached. We have found however that maleic acid fails to inhibit appreciably the activities of the lactic, glycerophosphate and malic enzymes, and the same is true—at least under the conditions of our experiments—of iodoacetic acid.

It seemed probable that substances which inhibit the succinic dehydrogenase must enter into such relations with the active structures of the enzyme as would make them capable of protecting it from oxidation. Malonic acid proved to be very effective in such protection. A tissue preparation exposed to GSSG and malonic acid is found, after being washed free from the acid, to have lost none of its original activity. The protection is exerted with very low concentrations (Exp. 10), much lower than those necessary for the inhibition of the succinic dehydrogenase.

Other efficient protectors are the dicarboxylic acids, succinic acid itself, for instance, and fumaric acid. This is in keeping with what is known as to the nature of the compounds which most readily inhibit the enzyme: two acidic groups being usually necessary. Pyrophosphoric acid also protects with somewhat less efficiency. It is of interest to know that Rapkine (personal communication), studying the inhibition of yeast fermentation by iodoacetic acid, has found that the above-mentioned dicarboxylic acids (though also lactic and glycollic acids) prevent this inhibition. He has come to the conclusion that this is because they may protect thiol groups which at some stage are essential for the fermentation process.

As bearing further on the proof that SH groups are essential to the activity of the succinic enzyme we endeavoured to obtain direct evidence that these groups remain intact when preparations are protected on the above lines. This again is made difficult by the circumstance that these groups, as present in the indifferent tissue proteins, are so greatly in excess of those which can belong to the enzyme itself. Some evidence for such protection which seems satisfactory has however been obtained and is described above.

With the evidence now available the significance of thiol groups in the activity of the succinic dehydrogenase scarcely admits of a doubt. In this respect the enzyme stands alone among the typical dehydrogenases so far studied, and it seems probable that its case is unique. It is remarkable that it thus shares a character with certain hydrolases while differing from enzymes with functions analogous with its own. This will add interest to future endeavours to discover the mechanism of its action.

SUMMARY

While the succinic dehydrogenase is inactivated by incubation with GSSG and its activity restored by subsequent incubation with GSH this treatment has no effect on the other dehydrogenases studied.

Alloxan in low concentrations, as an oxidant of SH-groups, inactivates the succinic enzyme but not the other dehydrogenases.

The succinic enzyme is inactivated by Cu and by maleic and iodoacetic acids; substances which react with SH groups. They have little or no effect on the other enzymes tested.

Malonic, succinic and fumaric acids protect the succinic dehydrogenase from the influence of GSSG.

The experiments described support the view that the succinic dehydrogenase requires for its activity the integrity of SH groups in its structure while this is not the case with other typical dehydrogenases.

When muscle tissue is exhaustively extracted with salt solutions dehydrogenases remain active in the residual structure of the fibres, which still shows cross striation and birefringence.

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Note added on 10 October, 1938. After the paper had been read in proof our attention was called to a publication by v. Euler and Hellström (*Ark. Kemi Min. Geol.* 1938. **13** B, 1) in which it is shown that oxidation by ferricyanide reduces the activity of the succinic dehydrogenase which reduction by hydrosulphite increases. This paper has now appeared in the current number of *Hoppe-Seyl. Z.* (**255**, 159).

CCXXXIX. STUDIES IN DETOXICATION

II. (a) THE CONJUGATION OF ISOMERIC 3-MENTHANOLS WITH GLUCURONIC ACID AND THE ASYMMETRIC CONJUGATION OF dl-MENTHOL AND dl-isoMENTHOL IN THE RABBIT. (b) d-isoMENTHYLGLUCURONIDE, A NEW CONJUGATED GLUCURONIC ACID

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WHEN terpenes (or their derivatives) are administered to animals, a considerable proportion is excreted as hydroxy derivatives conjugated with glucuronic acid; for example, 80 % of borneol is conjugated in this way in man [Pryde & Williams, 1936]. It was from the conjugated glucuronides formed after the feeding of camphor to dogs that *d*-glucurone was first isolated [Schmiedeberg & Meyer, 1879; cf. Jaffe, 1878]. Conjugation with glucuronic acid does not, however, account for all the terpene administered and it is probable that some portion undergoes complete oxidation. This work deals only with the conjugation.

The main objects of the present investigation were to find out whether the optical (enantiomorphic) and geometrical isomerism of the menthols (3-menthanols) influenced their conjugation with glucuronic acid in the body and whether the feeding of a *dl*-menthol resulted in the excretion of a conjugated glucuronide containing more of one antipode than the other.

The conjugation of menthol and isomenthol

It has already been shown by Quick [1932] and by Williams [1938] that conjugation can be influenced by position isomerism, i.e. *o*-, *m*- and *p*-substitution. The terpene alcohols, the menthanols, exhibit three types of isomerism, namely structural (e.g. 2-menthanol and 3-menthanol), geometrical (e.g. menthol and *iso*menthol) and optical isomerism (e.g. *d*- and *l*-menthol). The present investigation deals with the 3-menthanols alone. Four[1] of these are theoretically possible, each occurring in *d*-, *l*- and *dl*-forms [Simonsen, 1931]. Only two have been available and these are menthol (I and II) and *iso*menthol (III). The forms used were *l*- and *dl*-menthol and *d*- and *dl-iso*menthol.

Enantiomorphs   Geometrical isomerides
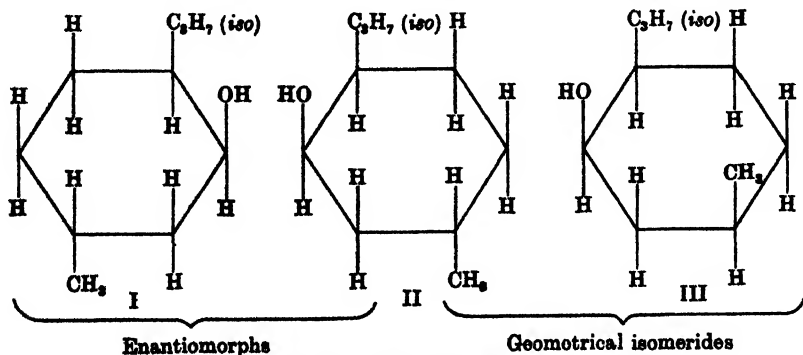
[1] Menthol, *neo*menthol, *iso*menthol and *neoiso*menthol.

The mean percentages of these substances conjugated with glucuronic acid in the rabbit under standard conditions (dose 1 g./kg.) are summarized in Table I. The calculated values for *d*-menthol and *l*-isomenthol are also shown.

Table I. *The percentage conjugation of menthol and* isomenthol *with glucuronic acid*

Menthol	% conjugated	isoMenthol	% conjugated
l-	48	*l*-	45 (calc.)
dl-	59	*dl*-	55
d-	70 (calc.)	*d*-	65

Table I shows that there is only a slight difference between the conjugation values of menthol and *iso*menthol provided that similar forms are compared, but there is a pronounced difference between those of their optical antipodes. The conjugation of either of the *d*-antipodes is nearly 50 % greater than that of either of the *l*-antipodes. The conclusion is therefore drawn that the conjugation of these two menthanols with glucuronic acid in the rabbit is little influenced by their geometrical isomerism but considerably affected by their optical isomerism.[1] Further, the menthanols possessing the *d*-configuration are more easily conjugated and therefore presumably more resistant to biological oxidation than those of the *l*-configuration.

The asymmetric conjugation of dl-menthol *and* dl-isomenthol

Since one optical isomeride is conjugated to a greater extent than its enantiomorph, it follows that on feeding an optically inactive *dl*-mixture, the excreted glucuronide should contain more of one isomeride than of the other. It has already been shown qualitatively that after feeding *dl*-borneol, *dl*-isoborneol [Hämäläinen, 1909], *dl*-menthol [Jacobsohn & da Cruz, 1929: 1930] or *dl-p-sec*-butylphenol [Fromageot, 1929], these compounds are excreted in conjugated forms which contain unequal amounts of the original *d*- and *l*-compounds. Administration of *dl*-camphor also results in the excretion of an optically active campherol [Meyer, 1908], but in this case the result may be complicated by the fact that at least two camphcrols (3- and 5-hydroxycamphors) are formed after the feeding of camphor to dogs [Asahina & Ishidate, 1928]. It has also been shown [Pryde & Williams; 1934] that when commercial *d*-borneol is fed to dogs, the borneol excreted in conjugation with glucuronic acid contains a greater proportion of the *d*-isomeride than that fed. None of this work was quantitative, since no estimations were made of the extent of conjugation of either of the optical antipodes or of the *dl*-compound itself. Further, conjugated glucuronides such as *d*-menthyl-β-*d*-glucuronide and *l*-menthyl-β-*d*-glucuronide, are not optical antipodes and therefore fractional crystallization is possible during the isolation of the mixed glucuronides after the feeding of a *dl*-compound. Hence the amount of each isomeride in the compound isolated may depend to some extent on a difference in solubility between the two glucuronides. Table I shows that *d*-menthol is conjugated to greater extent than *l*-menthol; if *dl*-menthol behaves in the body as a simple mixture of equal proportions of *d*- and *l*-menthol, then the excreted glucuronide after feeding *dl*-menthol should contain more

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[1] The term "optical isomerism" is here used in the limited sense of "enantiomorphism". All the geometrical isomerides of 3-menthanol possess, in addition to a cyclic structure, three asymmetric carbon atoms and are optically active. They are therefore, broadly speaking, optical isomerides, but they are are not all enantiomorphs.

d-menthol than l-. From the figures in Table I it can be calculated that the menthol recovered after the administration of dl-menthol should have $[\alpha]_D$ about $+10°$ in alcohol (taking menthol as $[\alpha]_D \pm 50°$ in alcohol), i.e. about 60 % d- and 40 % l-. In duplicate experiments values of $+14\cdot4°$ and $+12\cdot8°$ (in alcohol) were obtained and these figures correspond to d- 64 %, l- 36 % and d- 63 %, l- 37 % respectively. A similar argument can be applied to dl-isomenthol, the calculated value of the rotation being about $+5°$ (in alcohol) which corresponds to 59 % d- and 41 % l- (isomenthol has $[\alpha]_D \pm 26°$ in alcohol). The figures found experimentally with isomenthol were $+6\cdot3°$ (i.e. 62 % d- and 38 % l-) and $+5\cdot8°$ (i.e. 61 % d- and 39 % l-). The optical measurements are therefore in fair agreement with values calculated from the chemical analyses quoted in Table I (see also Table III).

d-isoMenthyl-β-d-glucuronide

During the progress of this work a new conjugated glucuronic acid has been isolated from the urine of rabbits following the administration of d-isomenthol. The probable structure of this product (d-isomenthylglucuronide) is shown in the accompanying formula (IV). A description of the acid and some of its derivatives is given in the experimental section.

IV
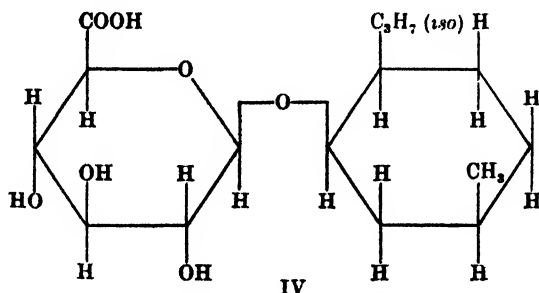
It has also been found that dl-menthol and dl-isomenthol give good yields of their respective glucuronides when fed to rabbits. This suggests their possible use as profitable biosynthetic sources of glucuronic acid [cf. borneol, Quick, 1927].

EXPERIMENTAL

Female rabbits, fed on a diet of 100 g. cabbage and 50 g. bran a day, were used under conditions similar to those already described [Williams, 1938]. dl-isoMenthol,[1] l- and dl-menthols were fed in warm water emulsions and d-isomenthol in aqueous suspension by stomach tube. The urine was collected for 2 days and analysed for conjugated glucuronic acid by ether extraction in a small continuous extractor (extracting 10 ml. urine in 3 hr.). The ethereal extract was evaporated and the residue hydrolysed by acid and then neutralized. The free glucuronic acid was estimated with Benedict reagent by the Quick method [1925]. The Benedict reagent was standardized against analytically pure d-isomenthylglucuronide, $C_{16}H_{28}O_7, H_2O$, and glucose. The ratio of the amounts of glucuronic acid and glucose required to reduce a given volume of Benedict reagent was $1\cdot098$ (calc. $1\cdot077$). The experimental results for the percentage conjugations of the 3-menthanols are given in Table II, whilst the average figures are given in Table I.

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[1] Rotations: $[\alpha]_D$ in alcohol: l-menthol (B.D.H.), $-50\cdot6°$ $(c=1\cdot4)$; d-isomenthol (Howard, Ilford), $+25\cdot2°$ $(c=1\cdot0)$; dl-menthol (Howard, Ilford), $0°$; dl-isomenthol (Howard, Ilford), $0°$.

Table II. *The percentage conjugations of menthol and isomenthol with glucuronic acid*

Substance	Rabbit no.	Wt. of rabbit (kg.) and dose (g.)	Urine vol. ml.	Glucuronic acid g.	Menthol conjugated g.	Menthol conjugated %
l-Menthol*	2	2	224	0·996	0·801	40
	5	2·4	357	1·393	1·120	47
	6	2·4	358	1·582	1·273	53
	2	2	265	1·345	1·081	54
dl-Menthol	1	2	248	1·514	1·217	61
	2	2	199	1·458	1·173	59
	3	2	254	1·444	1·161	58
*d-iso*Menthol	1	2	224	1·743	1·402	70
	2	2	273	1·567	1·260	63
	1	2	307	1·511	1·214	61
*dl-iso*Menthol	2	2	314	1·484	1·194	60
	1	2	216	1·364	1·097	55
	1	2	190	1·226	0·986	49

* In two experiments with *l*-menthol Quick [1924] found values of 42 and 47·5%, the dose being 2 g.

Experiments on asymmetric conjugation

(a) dl-*Menthol*. A rabbit was given 3 g. of *dl*-menthol in warm water by stomach tube and the urine collected for one or two days (see Table III). The urine was made slightly alkaline with NH_4OH and the precipitate of phosphates removed. To the filtrate solid $(NH_4)_2SO_4$ (50 g./100 ml.) was added and the whole boiled and filtered. The filtrate was kept in the refrigerator overnight. The crystalline precipitate of ammonium menthylglucuronate was separated, dried *in vacuo* and weighed. This salt was then hydrolysed by boiling under reflux for 1 hr. with 10% H_2SO_4. The hydrolysed solution was steam-distilled and the distillate cooled in ice. The solidified menthol was collected from the distillate, carefully dried with filter paper and weighed. The M.P. of the recovered menthol was 25–28°. Its specific rotation was measured in absolute alcohol (see Table III).

Table III. *The recovery of menthol and isomenthol from urine after the feeding of their dl-forms*

Rabbit no.	Substance fed	Dose g.	Urine vol. ml.	Menthol recoverable g.	Menthol recovered in NH_4 salt g.	Menthol from steam distillation g.	$[\alpha]_D$	$[\alpha]_D$ calc.
6	*dl*-Menthol	3	202*	1·77 (59%)	1·6	1·5	+14·4°	+10°
3		3	300†	1·77 (59%)	1·5	1·3	+12·8°	
4	*dl-iso*Menthol	2·5	222*	1·4 (55%)	1·2	1·0	+6·3°	+ 5°
5		3	197*	1·7 (55%)	1·7	1·6	+5·8°	

* In 1 day.   † In 2 days.   Rotations in absolute alcohol.

(b) dl-iso*Menthol*. The procedure used was the same as for *dl*-menthol except that the filtration after adding $(NH_4)_2SO_4$ was omitted. This omission was necessary since ammonium *iso*menthylglucuronate would not completely redissolve in the boiling urine after the addition of $(NH_4)_2SO_4$. The recovered *iso*menthol had M.P. 40–42° (see Table III).

d-iso*Menthyl-β-d-glucuronide and its derivatives*

*d-iso*Menthylglucuronide can be isolated from urine directly as the free acid or, after precipitation with $(NH_4)_2SO_4$, as the ammonium salt.

(a) *Isolation as free acid.* The isolation of the free acid is an exceedingly simple procedure, since the compound is precipitated from the urine when the latter is acidified. Rabbits were given 2–3 g. of *d-iso*menthol with water by stomach tube. The excretion of the glucuronide was practically complete within 24 hr. The urine was collected after one or two days and, after filtering through cotton wool, was made strongly acid with 20 % (wt./wt.) H_2SO_4 (25 ml. acid to 100 ml. urine). Warming to 40° accelerates the separation. The glucuronide began to separate immediately and the process was complete after several hours in the refrigerator. The compound was then filtered under suction, washed with water and dried. In most cases the crude glucuronide had a pinkish colour due to urinary pigments, but in some cases an almost pure white product was obtained. One recrystallization from hot water (charcoal) gave a white crystalline product, M.P. *ca.* 120°. The yield was 0·7 g. per g. of *d-iso*menthol fed (see Table IV).

(b) *Isolation as ammonium salt.* The urine was made slightly alkaline with ammonia, boiled and the precipitate of phosphates removed by filtration. To the filtrate solid $(NH_4)_2SO_4$ was added (30 g./100 ml.) and the solution boiled. Immediate precipitation of the ammonium *d-iso*menthylglucuronate took place and the process was completed in the refrigerator. The crude salt was then separated and dried. The yield was 1·2 g. per g. of *d-iso*menthol fed. The recovery of material was better than by the first method (see Table IV).

Table IV. *The recovery of* d-isomenthylglucuronide *from urine*

Rabbit no.	Dose g.	Yield as free acid g.	Rabbit no.	Dose g.	Yield of NH₄ salt g.
1	2	1·6	5	2·5	3·0
1	2	1·4	6	2·5	2·8
2	2	1·0	7	2·5	3·2
4	2·5	1·75			
6	2·5	1·8			

d-iso*Menthyl-β-d-glucuronide.* The crude glucuronide after two recrystallizations from a large volume of hot water was obtained pure. It forms long fine matted needles, M.P. 126° (corr.); it is very sparingly soluble in cold water, but easily soluble in hot water, ethyl alcohol, methanol and ether. It crystallizes from water with $1H_2O$ and has $[\alpha]_D^{16°} -43·2°$ ($c = 1·2$ in abs. alcohol). (Titration: 114·75 mg. required 16·48 ml. of 0·0198 N NaOH; calc. 16·55 ml. Found: C, 54·75; H, 8·6; H_2O, 5·0 %. $C_{16}H_{28}O_7$, H_2O requires C, 54·8: H, 8·6: H_2O, 5·1 %.)

Ammonium d-isomenthyl-β-d-*glucuronate.* The crude ammonium salt was purified by dissolving in hot water, filtering and precipitating with $(NH_4)_2SO_4$. Three such treatments gave an analytically pure salt as glistening plates. The salt was anhydrous and had $[\alpha]_D^{18·5} -41·1°$ ($c = 1·3$ in water). (Found: N, 4·02 %. $C_{16}H_{31}O_7N$ requires N, 4·01 %.)

Cadmium d-isomenthyl-β-d-*glucuronate.* Aqueous solutions of the ammonium salt gave amorphous precipitates with soluble Cu, Fe, Zn, Ag and Co salts. On adding aqueous $CdCl_2$ to a solution of the above ammonium salt and slightly warming the mixed solutions, an insoluble crystalline cadmium salt separated as shining platelets. (Found: Cd, 14·7 %. $(C_{16}H_{27}O_7)_2$ Cd requires Cd, 14·5 %.)

Hydrated cadmium salts of *l*-menthylglucuronide have been prepared by Fromm & Clemens [1901], but the present compound was anhydrous and lost no weight at 110°.

The recovery of conjugated glucuronic acid after the feeding of dl-*menthol* and dl-isomenthol

By using the $(NH_4)_2SO_4$ precipitation method already described, good yields of crude ammonium menthyl- and *iso*menthyl-glucuronates can be obtained from the urines after feeding the *dl*-compounds. The yields were 1·1 to 1·4 g. per g. of the *dl*-menthanols fed (see Table V).

Table V. *Yields of glucuronides after* dl-*menthol and* dl-isomenthol

Exp.	Substance	Dose g.	Yield of crude NH₄ salt g.
1	*dl*-Menthol	3	4·1
2	,,	3	3·3
3	,,	2	2·5
4	*dl-iso*Menthol	2·5	3·0
5	,,	3	4·4

These ammonium salts are of course mixtures containing more of the glucuronides of the *d*- than of the *l*-isomerides.

The glucuronide after feeding dl-*menthol*. Some of the crude ammonium salt obtained after feeding *dl*-menthol was further purified and converted into the free acid by adding the requisite amount of dilute HCl. The free acid was recrystallized from hot water, crystallization being accelerated by adding a little dilute HCl to the cooled solution. One purified specimen had M.P. 103–104° and $[\alpha]_D^{19°} -41\cdot8°$ ($c=2$ in alcohol) (pure *l*-menthylglucuronide has $[\alpha]_D -105°$ in alcohol [Fischer, 1910]). Like the pure *l*-derivative this mixture crystallizes with 1·5 H_2O. (Titration: 108·6 mg. required 15·16 ml. of 0·01994 N NaOH; calc. for $C_{16}H_{28}O_7$, 1·5 H_2O, 15·17 ml.)

The glucuronide after dl-isomenthol. A specimen of the crude ammonium salt recovered after the feeding of *dl-iso*menthol was subjected to two further recrystallizations from hot half-saturated $(NH_4)_2SO_4$ solution. The purified material had $[\alpha]_D^{18°} -54\cdot4°$ ($c=1\cdot6$ in water); pure ammonium *d-iso*menthyl-glucuronate has $[\alpha]_D -41°$ (in water).

It must be pointed out, however, that *d*- and *l*-menthyl- (or -*iso*menthyl-) glucuronides are not optical antipodes and therefore the purification of the mixed free acids or ammonium salts will result in some measure of fractional crystallization. The measured specific rotations will therefore be somewhat variable. Experience gained in this work shows that the *l*-compounds are more soluble than the *d*-menthylglucuronides. The separation of the *l*- and *d*-menthyl-glucuronides is being further investigated.

SUMMARY

1. The extents of conjugation of the geometrical isomerides, menthol and *iso*menthol, with glucuronic acid in the rabbit are almost identical, provided that similar optical isomerides are compared. The optical isomerides (enantio-morphs), *d*- and *l*-menthol and *d*- and *l*-*iso*menthol show pronounced differences in conjugation, the *d*-compounds conjugating to nearly a 50 % greater extent than the *l*-.

2. Asymmetric conjugation with glucuronic acid takes place when *dl*-menthol and *dl-iso*menthol are fed to rabbits. The menthol and *iso*menthol recovered from the urine have positive rotations which agree with the values calculated from the percentage conjugations of the separate *d*- and *l*-isomerides.

3. The preparation and properties of *d-iso*menthyl-β-*d*-glucuronide and of two of its derivatives are described for the first time.

4. It is suggested that *dl*-menthol and *dl-iso*menthol can be used as profitable sources of biosynthetic glucuronic acid.

The *dl*-menthol, *d*- and *dl-iso*menthol used in this work were supplied by Messrs Howard and Sons, Ltd., Ilford.

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CCXL. STUDIES IN THE SECRETION OF MILK FAT

I. THE EFFECT OF INANITION ON THE BLOOD LIPOIDS OF THE LACTATING COW

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DURING the past few years the work of Lintzel [1934], Blackwood [1934], Graham *et al.* [1936] and Maynard *et al.* [1938], based on the principle of simultaneous arterial and venous blood sampling, has shown that the only lipoid fraction of the blood used by the mammary gland for milk fat production is the neutral glyceride fraction and not that comprising the phosphatides or cholesteryl esters. At first sight this might seem to suggest that for the secretion of milk fat the gland simply selects from the blood the particular type of glycerides which it requires and that these are then allowed to diffuse into the milk. But it is clear from the peculiar composition of the fat itself and also from the fact that the respiratory quotient of the actively lactating mammary gland has now been found by Graham *et al.* [1938] to be considerably greater than unity, that more complex processes such as the synthesis of fat from carbohydrates are also undoubtedly involved. It would therefore appear that there are at least two main precursors of milk fat in the blood, the neutral glycerides on the one hand and some form of carbohydrate material on the other, and so it seems reasonable to suppose that the composition of the milk fat produced by an individual animal at any particular time will depend on the extent to which each of these precursors contributes towards its production. It was felt that further evidence regarding the biochemical and physiological processes involved in milk fat secretion might be obtained by a detailed study of both the blood and milk fats of animals kept under such experimental conditions as would be expected to alter the proportions in which these two precursors were used by the gland. One possible method of achieving this end would be to cause in some way a marked alteration in the lipoid concentration of the blood of the cow in the hope that an increase or decrease in the level of triglycerides in the plasma would considerably alter the proportion of the milk fat arising from this particular fraction of the blood. Consequently, it was decided to investigate the problem by experiments involving inanition and certain other procedures which might be expected to cause a considerable alteration in the relative availabilities of the two precursors. The present paper and the communication which follows it are concerned only with the inanition work, the first dealing exclusively with the blood lipoids and the second with the milk fat. The effect of inanition on the general composition of the milk has already been discussed by Smith *et al.* [1938].

Experimental

The blood and milk fat samples analysed in the present work were obtained in the course of an experiment undertaken by Dr S. Morris to determine the effect of inanition on the metabolism of lactating and non-lactating ruminants. The animals used were three non-pregnant Ayrshire cows in the sixth to seventh month of their lactation period. After a few weeks, during which they had been receiving a normal well-balanced diet, food was withheld from two of them for a period of 12 days, after which free access to food was again permitted, water being given freely at all times. Under these circumstances Cow no. 1 appeared to present a case of simple inanition throughout the 12 days, but with Cow no. 2 severe symptoms of milk fever occurred in the second day of the fast, and although they rapidly disappeared after inflation of the udder, their occurrence must be taken into consideration as a possible complicating factor. For Cow no. 3 the experiment was discontinued on the seventh day of the fast because by that time the condition of the animal was not satisfactory, a fact which may well have been due to a marked deficiency in depot fat as revealed by subsequent *post mortem* examination. At suitable times before, during and after inanition, blood samples were taken from one of the mammary veins, and composite daily milk samples were collected for the isolation of the fat.

Extraction of the blood lipoids

About 200 ml. of blood were centrifuged for 45 min. and the plasma was separated from the corpuscles. The plasma was then allowed to drop in a fine stream into about 15 volumes of alcohol-ether (3 : 1), the amount of solvent made up to 20 volumes and the mixture left for a few hours at room temperature with occasional shaking. It was then filtered, aliquots of the filtrate were removed for the colorimetric estimation of phosphorus and a known volume of the filtrate, usually about 2 l., was reduced to dryness on a water bath *in vacuo* under an atmosphere of nitrogen. The residue so obtained was extracted several times with chloroform and the extracts were filtered from the relatively large amounts of blood inorganic salts which accumulated at this stage. The corpuscles were similarly treated except that they were first mixed with an equal volume of water to cause haemolysis and the mixture was finally made up in 25 volumes of alcohol-ether. 20 volumes of solvent were used for the plasma and 25 for the corpuscles, since it has recently been shown by Boyd [1936, 1, 2] that these are the minimum proportions which can be used with safety if the fat extraction in both cases is to be as complete as possible.

Analysis of the blood lipoids

Owing to the fact that the volume of blood available in most animal experiments and also the concentration of lipoids in the blood are both so exceedingly small, accurate analysis of the blood lipoids has always been a matter of considerable difficulty. In the present work, for reasons outlined later, it was decided not to employ any of the usual micro methods, but rather to take blood samples of such a size (about 200 ml.) that the ordinary standard macro procedures, suitably adapted for use with unusually small amounts, could be applied to the resulting lipoids. This was possible in the present work since the withdrawal of such large samples of blood is without ill effect on the cow. For every sample, therefore, fatty acids and total unsaponifiable matter were determined by direct weight, cholesterol by weighing the digitonide, iodine values by the
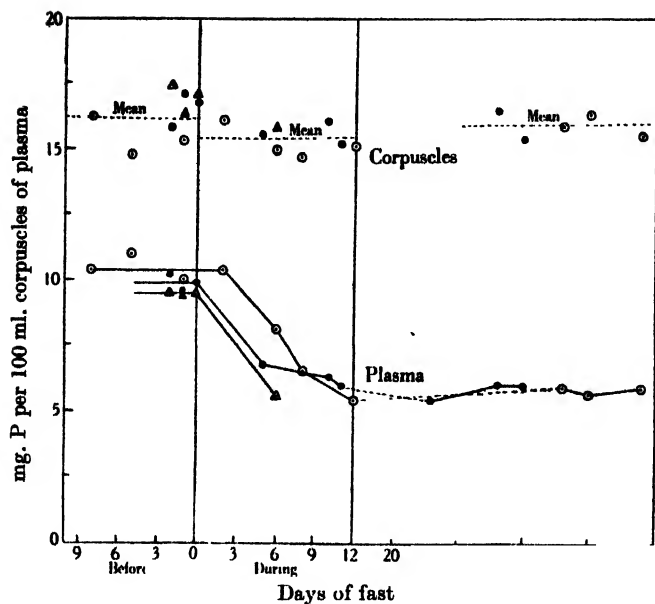
Fig. 1. The effect of inanition on the lipoid phosphorus content of the blood.
●—● Cow no. 1. ⊙—⊙ Cow no. 2. △—△ Cow no. 3.

Table I. *The analytical data for the corpuscle lipoids (mg./100 ml.)*

Days of fast		Total fatty acids			Total unsaponifiable matter	Cholesterol	Cholesterol content of unsaponifiable matter
		Wt.	I.V.	Mol. wt.			
				Cow no. 1			
Before	2	—	63	354	—	—	—
	1	374	60	360	227	183	81
	0	400	63	379	222	186	84
During	5	381	60	367	225	186	82
	10	406	59	349	222	192	86
	11	359	60	363	216	186	86
After	28	355	63	359	217	174	80
	30	363	62	342	188	165	88
				Cow no. 2			
Before	8	388	67	380	228	190	83
	5	404	65	363	207	180	87
	1	400	67	377	215	183	85
During	2	397	66	391	236	200*	85
	6	372	61	341	214	178	83
	8	386	56	—	220	176	80
	12	371	59	350	205	188	91
After	38	362	59	367	201	167	83
	40	378	62	340	213	171	80
	44	371	60	359	222	172	78
				Cow no. 3			
Before	2	432	64	336	207	196	95
	1	413	63	398	216	187	88
	0	408	64	334	206	193	94
After	6	389	59	364	214	196	92

* Day on which Cow no. 2 suffered from milk fever.

bromine absorption method and molecular weights by titration. Many precautions are necessary if accurate results are to be obtained, but as it is hoped that a separate communication on the details of the procedure will shortly be published, only the barest outline of the methods need be recorded here.

Corpuscles. As the corpuscle lipoids consist almost entirely of free cholesterol and phosphatides it was felt that nothing would be gained by the preliminary separation of the phosphatides. The solvent was therefore removed from the chloroform solution, obtained as already described, and the residue saponified directly by heating it for about 30 min. with 20 ml. alcohol and 2 ml. 40 % KOH, to which 20 ml. benzene were added to render the phosphatides more soluble. The fatty acids and unsaponifiable matter were then isolated, washed with water and finally made up to known volumes in light petroleum, from which suitable aliquots were taken for analysis. The results for lipoid P as estimated in the original alcohol-ether extract are shown in Fig. 1, while the remaining values are recorded in Table I.

Plasma. After the removal of the solvent from the chloroform solution of the plasma lipoids, the residue was dissolved in about 10 ml. of ether and transferred to a centrifuge tube and the phosphatides were precipitated with 30 ml. acetone. After standing at room temperature, the mixture was centrifuged, the clear supernatant liquid poured off and the residue washed with acetone. It was then dissolved in ether and reprecipitated. Finally, both the phosphatide and non-phosphatide fractions were made up to known volumes and analysed. That almost a complete separation of the two fractions had been obtained was indicated by the facts that the P content of the non-phosphatide fraction was not more than 3 % of the whole and was often considerably less and that no more than the merest traces of cholesterol remained in the phosphatides. For this precipitation the use of $MgCl_2$ and a temperature of $0°$ are frequently recommended in the literature in order to assist the separation of the phosphatides, but it was found in the present work that these extra details were unnecessary, provided that really pure ether and acetone were available, and, as there is a very serious risk of precipitating some of the cholesteryl esters and neutral glycerides along with the phosphatides at such a low temperature as $0°$, and as Mg ions may tend to cause difficult emulsions at a later stage, the simpler procedure outlined above was found to be the safest and most efficient.

After the P estimation, the phosphatide fraction was saponified as already described for the corpuscles. Total fatty acids and unsaponifiable matter were then isolated and analysed. For the non-phosphatide fraction, total lipoids and free cholesterol were first estimated in suitable aliquots before the remainder was saponified. The variations in concentration of the different plasma lipoids for the three animals are shown in Figs. 1, 2 and 3, while the molecular weights and iodine values of the acids are arranged in Table II.

One of the chief difficulties in analysing blood lipoids at the present time is that no method has yet been found for separating the cholesteryl esters from the neutral glycerides, these fractions, which together form the two main constituents of the non-phosphatide fraction, having very similar solubility properties. It is only possible to estimate the amount of fatty acids originally present as triglycerides by calculation. This was done here by assuming that the mean molecular weight actually found for the total non-phosphatide fatty acids was also the mean molecular weight of the acids esterified with cholesterol, which, as the latter greatly predominate, would give a very close approximation to the truth. From the amount of glyceride fatty acids so estimated it was possible to calculate the amount of glycerol which must have been required for
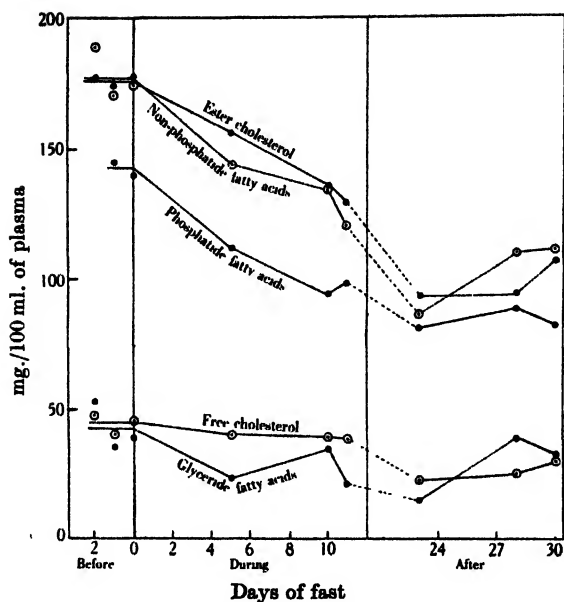
Fig. 2. The effect of inanition on the plasma lipoids of Cow no. 1.
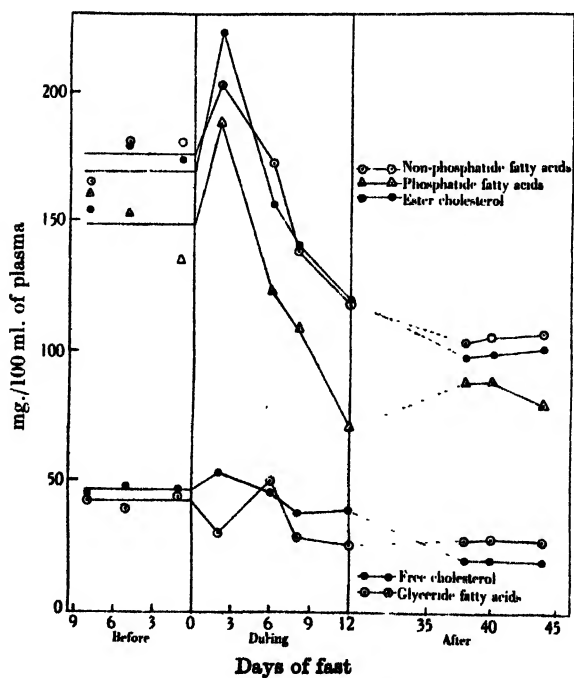
Fig. 3. The effect of inanition on the plasma lipoids of Cow no. 2.
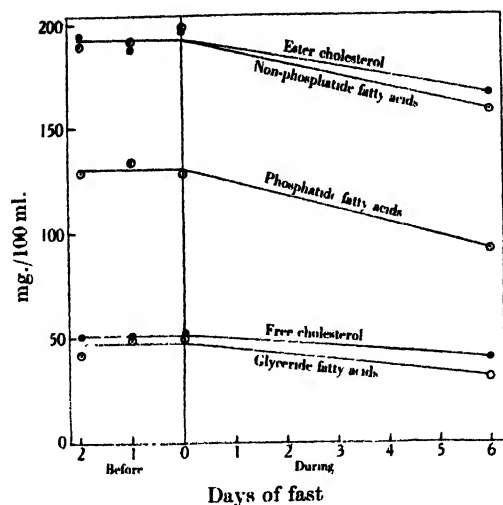
Fig. 4. The effect of 6 days' inanition on the plasma lipoids of Cow no. 3.

Table II. *Analytical data for the plasma lipoids not shown diagrammatically in the Figs.*

Days of fast		Fatty acids				Total cholesterol content of unsaponifiable matter %	Sum of fatty acids, un-saponifiable matter and glycerol as % of original non-phosphatide fraction
		Phosphatide		Non-phosphatide			
		I.V.	Mol. wt.	I.V.	Mol. wt.		
Cow no. 1							
Before	2	64	333	134	295	99	97
	1	66	359	136	295	94	95
	0	59	335	135	293	94	95
During	5	62	320	136	295	91	96
	10	65	340	125	304	96	94
	11	62	359	134	291	96	90
After	23	62	322	137	294	85	94
	28	74	323	129	294	91	94
	30	74	340	136	295	92	94
Cow no. 2							
Before	8	60	342	139	304	90	99
	5	71	331	142	305	96	99
	1	60	318	137	312	97	99
During	2	68	332	149*	306	97	97
	6	69	388	137	303	94	97
	8	70	333	140	304	91	99
	12	58	370	137	297	93	94
After	38	63	355	130	301	88	92
	40	57	347	131	302	90	94
	44	65	348	130	304	90	88
Cow no. 3							
Before	2	77	333	140	293	98	98
	1	69	323	143	289	97	96
	0	65	366	142	293	98	97
After	6	59	370	143	294	99	93

* Day on which Cow no. 2 suffered from milk fever.

their esterification. Then, by adding together the values found for total fatty acids, total unsaponifiable matter and those calculated for glycerol, it was possible to estimate the percentage of the original non-phosphatide fraction which was accounted for by the various constituents. It will be observed from Table II that this figure was usually well over 90%, a fact to which reference is made later.

DISCUSSION

The work of Maynard & McCay [1929] and of Porcher & Maynard [1930] has shown that the blood lipoid level of ruminants under normal circumstances changes extremely slowly and that variations in it tend to be but slight. In fact with non-lactating cows Aylward & Blackwood [1936] found no definite or consistent alteration in the total fatty acid content of the blood even after a fast of 10½ days, while later results of Aylward et al. [1937] showed that although for two days after a drench of "labelled" fat the blood of the cow became gradually enriched with the "labelled" constituents of the drench, the actual lipoid level itself was not measurably affected. It was therefore obvious that if the small changes in composition or in amount which may be expected in the blood fat of ruminants were to be detected, the methods adopted for analysis would have to be as searching and as accurate as possible. For this reason it was felt that the micro procedures now commonly in use such as that of Bloor [1928; 1929], were not applicable to the present experiments as they would not deal with sufficient material for the thorough examination which the author had in view. Nor was another type of method such as that published by Stoddart & Drury [1929] of much value here, as it depends on the assumption that the mean molecular weight of the fatty acids is always a given value from which their amount is calculated, whereas actually in the present work, the constancy or variability of these particular molecular weights before and during inanition was one of the properties which it was desired to investigate. It was therefore decided that the macro procedure, suitably adapted, would be the one most likely to give the best results. Although this method is somewhat long and laborious, it was believed that its application to a few large blood samples would be of much greater value than more numerous but less reliable analyses carried out on very much smaller amounts.

Phosphorus. The lipoid P results shown in Fig. 1 are typical of the general effect of inanition found for the other lipoid fractions of the blood. In the corpuscles the average figure of 16·2 mg. per 100 ml. for all three cows prior to the fast fell by less than 5% to 15·4 during inanition, and a few weeks after re-alimentation it had tended to return towards the original value. That it never quite reached the initial figure may well have been due in part to the presence of small amounts of plasma still remaining in the corpuscles, for the latter were not washed with saline prior to their extraction as it was felt that, by prolonging the process of separation unnecessarily, more errors might be introduced than were actually avoided and that any plasma still remaining in the corpuscles would be too small to invalidate the general conclusions in any way.

In the plasma very much greater changes were observed than in the cells. The mean initial figures of about 10 mg. were reduced by almost 40% to 6 mg. during inanition, and strangely enough this low level persisted even 6 weeks after free access to food was again permitted. In the work of Aylward & Blackwood [1936], the lipoid P level in whole blood was found in most cases to rise immediately after food was withheld rather than to fall, but this unexpected observation can now be explained by the facts that almost half the lipoid P in whole blood is

contained in the corpuscles, that its level in the corpuscles is not much affected by inanition and that the proportion of corpuscles in the blood as shown by Smith *et al.* [1938] increases from about 30 to over 40 % in the initial stages of starvation. It is therefore only later in the fast that an appreciable reduction in the phosphatide content of whole blood is noticeable. It is of interest to observe that the total decrease in the lipoid P of whole blood found for dry cows by Aylward & Blackwood [1936] was about 10 % as compared with the much larger figure of 25 % which can be calculated for lactating cows in the present work, notwithstanding the fact now well established that phosphatides are not precursors of milk fat.

Corpuscles. One of the main objects of analysing the lipoids of the corpuscles was to find whether their level or nature was materially affected by very abnormal conditions, for if no more than minute changes could be observed under such a prolonged period of inanition, it is highly improbable that they would ever change appreciably under any normal circumstances to which the cow might be subject. Consequently, it would be most unlikely that variations in milk fat secretion, whatever their cause, could ever be traced to changes or temporary peculiarities in the lipoids of the cells. If this were in fact found to be so, extension of the corpuscle side of the work in future experiments of this type would be quite unnecessary.

Before discussing the results it is essential to mention that the complete extraction of the lipoids from corpuscles, even by the procedure recommended by Boyd [1936, 2], is by no means easy, nor can it readily be done with identical results for different samples taken at the same time from the same batch of corpuscles. This difficulty can doubtless be attributed to the fact that the cells contain such extremely small amounts of very complex phosphatides in the presence of relatively large quantities of proteins, with which they may even be loosely combined. It is therefore not surprising to find considerable variation in the fatty acid levels before inanition as recorded in Table I, in spite of the fact that there was probably little actual change in the pre-fast period. There is, however, sufficient consistency to show that on the whole there was a reduction of some 6 or 7 % in the total fatty acids as a result of inanition and that this decrease persisted for at least 6 weeks after re-alimentation. Thus with Cow no. 2 an original mean of 397 mg. fell to 371 mg. by the 12th day of the fast and was still close to this value 40 days later. That it did not show any tendency to return towards the original figure in the post-fast period, as was the case with the corpuscle P, was again probably due, in part at least, to the presence of small amounts of plasma still remaining in the corpuscles. As these small residual amounts of plasma had a higher ratio of fatty acids to lipoid P than was the case for the corpuscles themselves, they would have a greater effect on the figures for corpuscle fatty acids than on those for the lipoid P.

With regard to the nature of the corpuscle fatty acids, the iodine values in Table I suggest a very slight reduction in the degree of unsaturation during inanition. Thus, the mean initial figures of 62, 66 and 64 for the three cows fell to 60, 59 and 59 respectively towards the latter part of the fast, and showed a slight tendency to return towards the normal values in the post-fast period, but it is doubtful if such small variations have any significance. Unfortunately, from the molecular weights found for the acids little can be concluded. It appeared during the course of the experiments that the estimation of these particular molecular weights by titration is by no means a simple matter and that this is not entirely due to the very difficult end points which were encountered. The figures obtained ranged from 334 to 391 and possess at least two points of

interest: firstly, the great and irregular variations, and secondly, the magnitude of the values as a whole. From other so far unpublished work by the present author, it may be assumed that the corpuscle phosphatides are much more complex than is generally realized, but even if their main constituents were C_{20}, C_{22} and C_{24} acids, the mean molecular weight of all the acids would not be expected to be much greater than about 340. In fact one experiment on a much larger scale than the present has suggested that the value for corpuscle phosphatide fatty acids from a pooled sample of bovine blood is somewhat under 330. It is hoped that future work on the nature of these phosphatides will throw more light on this aspect of the subject, but at present it can only be stated that the variations in molecular weights as quoted in Table I are probably due to the difficulty of isolating the substances in a pure state and of making the actual determinations, and not to any significant change in the acids themselves.

Turning now to the cholesterol and total unsaponifiable matter, it is again seen from Table I that the alteration in level during inanition was in all cases negligible, but 30 or 40 days after the fast the cholesterol appears to have been somewhat reduced. Thus, taking Cow no. 1 as an example, the pre-fast figure of about 185 remained almost unchanged at a mean of 188 during starvation but then fell by some 9 % to 170 in the post-fast period. The reason for the lack of change during the fast and the decrease following re-alimentation cannot at present be given, but taking the results as a whole and comparing them with those of the plasma, these changes in level may be regarded as exceedingly slight.

As the unsaponifiable portion of blood lipoids is frequently assumed to consist almost entirely of cholesterol it was of considerable interest to estimate the actual proportion of each sample which consisted of this compound. It is clear from Table I that for Cows nos. 1 and 2 the mean figure for the amount of cholesterol in the unsaponifiable matter was 85 % and for Cow no. 3, 92 %, but the individual samples showed too much variation amongst themselves for any conclusion to be drawn as to the effect of inanition upon them. It is clear, however, that in every sample of unsaponifiable matter there was an appreciable amount of material soluble in light petroleum not precipitated by digitonin and not altered by resaponification. From these results as a whole it may therefore be concluded that the corpuscle lipoids of the lactating cow are not readily inclined to alter either in their nature or in their concentration even during prolonged inanition.

Plasma. Unlike the corpuscles, the plasma showed large reductions in the level of the various lipoid constituents as a result of starvation. With Cow no. 1, for example, there was a decrease during the fast of about 26 % in ester cholesterol, an initial figure of 176 mg. falling to just below 130 mg., while in the post-fast period this value fell still further to 94 mg., a total decrease of 47 %. Similarly with the phosphatide and non-phosphatide fatty acids, original figures of 143 and 177 became 94 and 119 towards the end of the fast and diminished further after re-alimentation to mean values of 83 and 105 respectively. These figures represent a total reduction of 45 and 40 % in the phosphatide and non-phosphatide fatty acids between the pre- and post-fast periods. The fact that these values were actually lower some weeks after free access to food had been permitted than they were when food was completely withheld is of considerable interest, though no definite explanation can at present be given. It may be that on re-alimentation, fat was so rapidly being used for replenishing the animal's depleted storage depots and for milk secretion that the level in the plasma could not be maintained above this low figure.

With Cow no. 2 the effects of inanition were complicated on the 2nd and 3rd days by the occurrence of milk fever, which was found to be accompanied by hyperlipaemia. Thus, as shown in Fig. 3, there were increases in the ester cholesterol, phosphatide and non-phosphatide fatty acids of 32, 25 and 14 % respectively, initial values of 169, 149 and 178 increasing to 223, 198 and 203. After the symptoms of milk fever had disappeared, however, these increases were followed by considerable decreases analogous to those found for Cow no. 1. Thus for ester cholesterol the value of 223 fell to 117 by the last day of the fast and was reduced to 100 in the post-fast period, a total decrease equal to 41 % of the initial pre-fast figure. Similarly the phosphatide and non-phosphatide fatty acids fell to 70 and 118 by the end of the starvation period and were as low as 85 and 106 some weeks after re-alimentation had been established, figures which are equivalent to decreases of 44 and 40 % respectively between the initial and final values. Again in Fig. 4 (Cow no. 3) it is confirmed that inanition causes a definite decrease in the various fatty constituents of the plasma, but for this cow the decrease only amounted to about 13 % for ester cholesterol, 30 % for phosphatide fatty acids and 18 % for those from the non-phosphatide fraction by the 6th day of the fast when the experiment was discontinued. It is of interest to notice from the figures just cited that in all cases, whether the lipoid level rose or fell, the phosphatide fatty acids tended to be affected to a somewhat greater extent than the others, perhaps owing to a greater degree of metabolic activity on the part of these particular acids.

Further reference to Figs. 2, 3 and 4 shows that there is also a very definite reduction in free cholesterol and in triglyceride fatty acids as a result of inanition. For Cow no. 1, for example, initial figures of 45 and 43 respectively were reduced to 38 and 21 by the 11th day of the fast and to mean values of 25 and 29 in the post-fast period, final decreases which are equivalent to 44 and 33 % of the original pre-fast figures. The corresponding reductions in free cholesterol and glyceride fatty acids for Cow no. 2 were 59 and 36 %, and for Cow no. 3, after a fast of only 6 days, they were 22 and 35 % respectively. The values for glyceride fatty acids in both Figs. 2 and 3 appear to be characterized by greater irregularities than any of the other lipoid constituents, and although this may actually have been the case in the plasma, it may well have been due mainly to the fact that these values were obtained, as already described, by calculation from the figures found for free and total cholesterol and for total non-phosphatide fatty acids. Any unavoidable errors therefore in the estimation of these latter fractions may have combined in several cases to give somewhat greater errors in the relatively small values calculated for the triglyceride acids. This suggestion gains support from the fact that no such irregularities occurred in the values for free cholesterol, which are almost equally small but which were found by direct estimation.

One point of considerable interest which may be stressed at this stage and which is very obvious from Figs. 1–4 is the extraordinary tendency in the plasma of the cow for all the various lipoid constituents, with the possible exception of the triglycerides, to behave alike under different conditions. Thus in milk fever, the rise in phosphatide fatty acids was accompanied by similar rises in cholesteryl esters and free cholesterol, while again during simple inanition they all decreased in much the same way, with the result that the general composition of the total lipoids tended to remain the same although their level in the plasma changed. This tendency to a constant composition for the lipoids of cow's plasma is exceedingly important, for, if general, it means that in large scale experiments to discover, for example, whether there is any correlation between the characteristic blood

fat level of an animal and its milk fat production, it might be possible in some sections of the work to estimate only lipoid P and cholesterol, both of which can readily be determined, while the more laborious and less accurate estimation of the fatty acids could be omitted, at least in a preliminary survey.

With regard to the effect of inanition on the actual nature of the various lipoid fractions, it may be concluded, just as for the corpuscles, that there was extremely little change. For the phosphatide fatty acids, the iodine values and molecular weights recorded in Table II show too much variation amongst themselves for any definite conclusion to be drawn, except that, considered as a whole, they do not suggest any noticeable alteration as a result of fasting in the general type of fatty acids present. Here again, as with the corpuscles, considerable difficulty was experienced in estimating the molecular weights of these particular fatty acids, so that the variations shown for them are thought to be due mainly to the experimental difficulties involved rather than to changes in the acids themselves. Curiously enough no such difficulties were encountered in estimating the molecular weights of the non-phosphatide fatty acids, for which the values were much more consistent. Thus with Cow no. 1, for example, the molecular weights of the phosphatide acids varied between 320 and 359, whereas for those from the non-phosphatide fraction, they all fell within the limits of 291 and 304, 7 out of the 9 being between 293 and 295. Why there should be this particular difficulty with the acids from the blood phosphatides is not at present clear, but it is hoped that future work will help to elucidate the problem. It is interesting to observe that the non-phosphatide fatty acids of Cow no. 2 had consistently higher molecular weights than those of the other two cows, the mean value being 305 for no. 2 as compared with 295 and 292 for nos. 1 and 3. Some individual variation is obviously possible in this respect.

A study of the data given in Table II for the iodine values of the non-phosphatide acids again suggests that inanition must have caused very little change, if any, in the nature of the acids. It is only with Cow no. 2 that any slight alteration can be seen, for on the day when this animal suffered from milk fever the initial mean iodine value of 139 rose to 149. It then fell again to 138 during the latter part of inanition and ultimately to 130 some 6 weeks after re-alimentation. It would appear therefore that for this animal hyperlipaemia was accompanied by an increase in unsaturation, suggesting the mobilization of unsaturated acids in the emergency caused by milk fever, and that hypolipaemia in the post-fast period was accompanied by a decrease in unsaturation, but at the most these changes were small.

It is a well known fact that it is possible for the iodine values and molecular weights of two mixtures of fatty acids to be the same in spite of differences in the composition of the mixtures. Consequently, where possible, it is advisable, as a third check, to estimate the amounts of saturated acids in the mixtures by the Twitchell separation process. Owing to the small amounts of acids available in the various samples in the present work it was only possible to apply this separation on a micro scale to pooled samples of the plasma non-phosphatide acids from Cow no. 1. The saturated acids so estimated amounted to 11·2 % of the total acids before and during inanition and to 12·4 % in the post-fast period. These figures may possibly be a little lower than the true values, as they were obtained on very small amounts of material, but they confirm the absence of any appreciable change as a result of the fast.

The proportion of cholesterol in the unsaponifiable matter of the plasma was somewhat higher than for the corpuscles. For example, for Cow no. 2 the figure for the plasma samples varied between 88 and 99 % with a mean of 96, whereas

in the corpuscles the limits were 78 and 91 with a mean of 84. Only during the post-fast period of the experiment were any appreciable changes noticed in these particular figures for the plasma. Thus with Cows nos. 1 and 2, pre-fast means of 96 and 94 % both remained almost unaltered during inanition but fell later to 89 %. It can scarcely be said, however, that such small decreases are significant.

In Table II it will be observed that for each sample the non-phosphatide fatty acids and unsaponifiable matter determined by direct weight have been added to the small amount of glycerol calculated to be present (usually about 3 mg./100 ml. plasma), and the sum has been expressed as a percentage of the original non-phosphatide fraction. The great majority of the figures so obtained are found to fall between 94 and 99 % suggesting that the amount of water-soluble fatty acids in the non-phosphatide fraction of the plasma is exceedingly small, if, indeed, they occur there at all. This finding is not in any way at variance with that of Stewart & Hendry [1935] who were able to suggest by other means that the water-soluble fatty acids of whole blood did not exceed 3 % of the total fatty acids.

SUMMARY

1. The effect of inanition on the blood lipoids of three lactating cows has been investigated in detail as part of a scheme for the study of certain problems connected with milk fat secretion.

2. Only very slight decreases of considerably less than 10 % were observed for the lipoid levels in the corpuscles, during and after a 12-day fast, whereas for the plasma, general reductions of as much as 40 or 50 % took place and these low levels persisted even 6 weeks after free access to food had again been permitted.

3. Neither in the corpuscles nor in the plasma could any appreciable change be detected in the actual nature of the lipoids as a result of inanition, although there was a tendency for greater unsaturation in the plasma fatty acids to accompany milk fever symptoms.

4. It was shown that if any water-soluble fatty acids are present in the non-phosphatide fraction of the plasma of lactating cows the actual amount must be exceedingly small.

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CCXLI. STUDIES IN THE SECRETION OF MILK FAT

II. THE EFFECT OF INANITION ON THE YIELD AND COMPOSITION OF MILK FAT

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IN the course of research work on the secretion of milk fat, the effect of inanition on the lipoids of both the blood and the milk of actively lactating cows has been studied in detail. The previous paper records the experiments on the blood lipoids, while the present communication describes the changes brought about in the fat of the milk during the fast. At the same time certain modifications are described for the analysis of butter fat by the fractional distillation process.

EXPERIMENTAL

The yield of fat was estimated daily by noting the yield of milk and determining its fat content by the Gerber process. The results are recorded in Fig. 1.
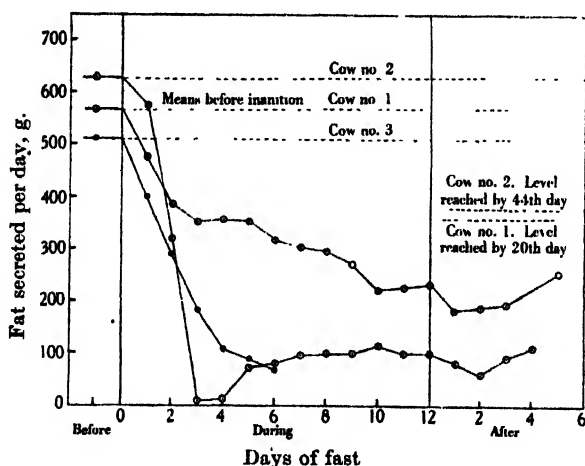
Fig. 1. The effect of inanition on the total daily yields of milk fat. The extremely low yield from Cow no. 2 on the 3rd and 4th days was due mainly to the incidence of milk fever.

For the determination of iodine and Reichert Meissl values, small samples of the milk fat were prepared daily by centrifuging some of the milk, chilling the resulting product in the refrigerator and carefully removing the cake of fat so formed from the top of the aqueous phase. The fat was then further purified by suitable treatment with light petroleum. The larger samples essential for fractional distillation were prepared by separating the milk, churning the

cream and purifying the fat finally by filtration. To ensure that the sample obtained was truly representative of the whole, tests were always made to ensure that the aqueous phase never contained more than the merest trace of fat. The figures for the iodine and Reichert Meissl values are shown diagrammatically in Fig. 2, where very regular changes can be observed for the fat secreted by each of the three cows during the fast.
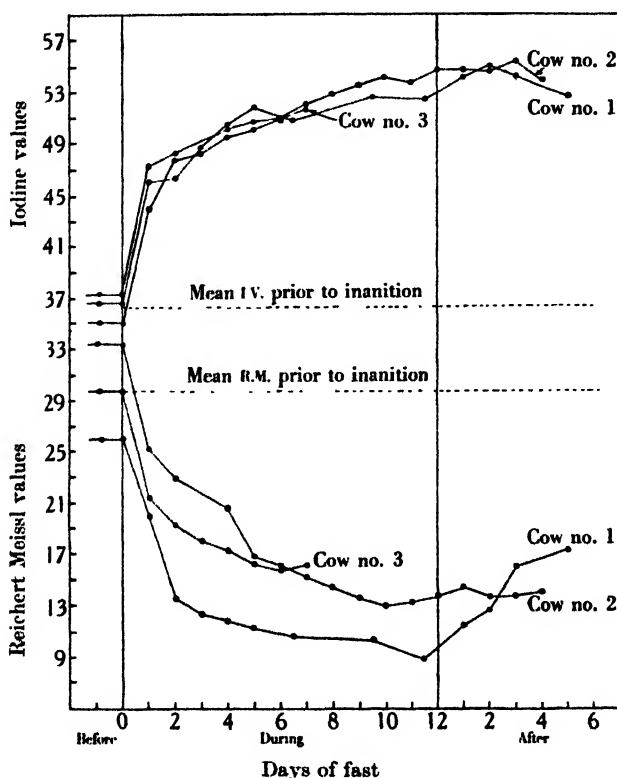
Fig. 2. The effect of inanition on the iodine values and
Reichert Meissl values of the milk fat.

Analysis by fractional distillation. Owing to the complex nature of butter fat the analysis of its component fatty acids has always presented some difficulty. Two main procedures have been used. Hilditch & Jones [1929] and Hilditch & Thompson [1936] first saponify the fat and then distil the acidified products for some hours in a current of steam. This is followed by the fractional distillation of the steam-volatile compounds, while the non-volatile residue is separated by the Twitchell process into solid and liquid acids. These are then converted into methyl esters and fractionated. Owing to other sources of error which seem to be unavoidable in any process of this type which can at present be devised, this procedure probably gives results which are as accurate as possible, but the present authors believe that there are slight disadvantages in the actual technique. One of these lies in the fact that during steam distillation, the non-volatile fatty acids have to be heated to about 100° in the presence of steam for several hours, a process which ought to be avoided when unsaturated acids are

present; another is that, unless very large quantities of the original butter fat are available, the amount of volatile compounds resulting from the steam distillation must be exceedingly small for their accurate fractionation. In the modified method now suggested distillation in steam is dispensed with, and the lower acids can be estimated more readily on smaller amounts of initial material. Bosworth & Brown [1933] also avoided the use of steam distillation in making a detailed investigation of the lower components of butter fat and were thus able to establish the presence of decenoic and tetradecenoic acids, but they did not adapt the method to the complete analysis of the total fatty acids. To do this the Twitchell separation of liquid and solid acids must be introduced at some stage in the process. The scheme which was therefore adopted in the present work may be summarized briefly by saying that the fat was converted directly into methyl esters, the lower components were fractionally distilled from the whole bulk, the higher members being separated into liquid and solid acids, methylated and also fractionally distilled. In order to explain the method more fully reference will be made in detail to the analysis of a typical butter fat, that secreted by Cow no. 1 prior to starvation.

The analysis of butter fatty acids by the modified method. The butter fat (about 287 g., Reichert Meissl value 26, i.v. 36·6) was first refluxed in 2 l. methyl alcohol containing 5 % H_2SO_4 for 24 hr. to convert the glycerides into methyl esters. Most of the alcohol was then boiled off and kept, while the esters were exhaustively extracted with ether and the united ether extracts washed three times with a little water. The ether solution was desiccated over sodium sulphate, filtered and the solvent almost completely removed in the water bath at ordinary pressure. The flask containing the esters was then connected to an electrically heated and packed fractionating column similar to that described by Longenecker [1937] and the last traces of solvent were carefully removed and collected under a very slight vacuum. Saponification values were then obtained for the methyl alcohol and ether was distilled from the esters at the two different stages, while the united aqueous portions were saponified with alkali, acidified with H_2SO_4, steam-distilled and the distillate was titrated. As all the methyl esters higher than the butyrate are insoluble in water it is reasonable to assume that all the acidity present in the various solvents was due to butyric acid. The amount of this compound present in the original fat could thus be calculated. A blank determination was carried out with a similar quantity of lard for which the Reichert Meissl value was negligible. The amounts of "butyric acid" estimated to be present for both the milk fat and the lard were 0·09 and 0·03 % in the aqueous portion, 2·73 and 0·03 % in the methyl alcohol and 0·68 and 0·25 % in the ether, making totals of 3·5 and 0·31 % of the butter and lard respectively. It is evident from these results that the estimated figure for butyric acid by this method tends to be somewhat high.

In the fractional distillation which followed, details of which appear in Table I, the first two fractions passed over at a pressure of 15 mm. and were kept separate. A large primary fraction containing all the esters distilling below 158–160° at 2 mm. was then collected, for it had been shown by preliminary trials that practically all the lower compounds up to and including most of the myristate pass over below that point, while the distillation of oleate is still relatively slight. This large primary fraction was then distilled into some 20 smaller fractions for which the iodine values and molecular weights were determined, and as these data suggested that the last fraction and residue consisted mainly of palmitate and oleate, these two final fractions were added again to the higher compounds still remaining undistilled. All these higher esters were

Table I. *The fractionation of the methyl esters prepared from 287 g. of the normal butter fat secreted by Cow no. 1 prior to inanition*

Lower esters

Fraction	B.P. at 2 mm. up to	% of total esters	Mol. wt.	I.V.	Fraction	B.P. at 2 mm. up to	% of total esters	Mol. wt.	I.V.
1	(Methyl butyrate)	3·88	—	—	12	118	0·76	216·4	9·1
2	45*	0·35	132·1	0·6	13	124	1·05	232·4	11·6
3	45*	0·32	131·8	0·6	14	127	1·12	233·4	10·8
4	56	0·22	158·5	2·6	15	127	1·61	238·3	10·7
5	56	0·42	157·4	2·7	16	128	2·38	239·8	10·4
6	56	0·07	159·2	—	17	131	1·66	242·1	9·1
7	70–80	0·56	177·8	12·8	18	141	1·31	248·4	9·0
8	82	0·67	184·8	16·5	19	145	1·56	260·9	8·9
9	84	0·61	183·1	16·4	20	148	3·44	266·2	9·5
10	102	0·51	195·5	12·9	21	149	1·76	269·5	11·3
11	106	0·94	209·5	7·9	Total		25·20		

Solid esters

Fraction	B.P. at 2 mm. up to	% of total esters	Mol. wt.	I.V.	Fraction	B.P. at 2 mm. up to	% of total esters	Mol. wt.	I.V.
22	140	0·45	245·0	0·7	29	162	3·16	281·0	8·2
23	149	0·67	262·3	0·4	30	158	3·25	283·2	9·0
24	153	1·61	268·4	1·3	31	161	2·73	284·0	9·8
25	153	2·38	274·4	4·0	32	165	2·92	289·2	11·2
26	159	2·93	274·5	4·1	33	165	1·93	290·2	13·7
27	157	3·47	277·8	5·0	34	170	2·69	297·3	13·9
28	161	3·20	278·0	5·6	35	Residue	3·05	307·7	17·9
					Total		34·44		

Liquid esters

Fraction	B.P. at 2 mm. up to	% of total esters	Mol. wt.	I.V.	Fraction	B.P. at 2 mm. up to	% of total esters	Mol. wt.	I.V.
36	132	0·30	237·2	17·1	44	163	3·27	287·8	77·6
37	132	0·46	244·2	17·4	45	164	2·76	288·7	79·9
38	145	0·37	254·9	27·4	46	165	2·78	290·5	82·1
39	153	0·60	268·9	46·3	47	166	4·72	295·1	85·9
40	155	1·20	281·5	67·9	48	168	4·91	295·3	87·4
41	155	2·40	282·2	72·2	49	168	4·26	295·5	88·7
42	161	2·18	284·7	72·4	50	170	4·47	294·8	90·3
43	163	2·40	287·1	76·6	51	Residue	3·28	303·1	93·1
					Total		40·36		

* At 15 mm. pressure.

then saponified and separated into liquid and solid acids and the liquid portion was freed from any small amounts of unsaponifiable matter which might be present. They were then esterified again and fractionally distilled through the electrically heated column. The iodine values, molecular weights and other data for the various fractions are recorded in Table I, the molecular weights being obtained either from the saponification values of the esters or by titration of the acids prepared from them. Corresponding data were then obtained for samples of starvation fat secreted by Cows 1 and 2, but in order to illustrate the marked differences between ordinary fat and that secreted during inanition, only the figures for the first of these need be recorded as shown in Table II.

Before the composition of the various fractions could be calculated, according to the method adopted and discussed by Irving & Smith [1935], it was first necessary to oxidize several fractions to ensure that the assumptions made as to their actual nature were justified. There is, however, no necessity to describe these oxidations in detail. In order to show the type of fractionation obtained without the use of steam distillation, the intermediate results obtained for the composition of the three main fractions, the lower, solid and liquid esters of both

Table II. *The fractionation of the methyl esters prepared from 285 g. of the butter fat secreted by Cow no. 1 on the 11th and 12th days of inanition*

Fraction	B.P. at 2 mm. up to	% of total esters	Mol. wt.	I.V.	Fraction	B.P. at 2 mm. up to	% of total esters	Mol. wt.	I.V.
					Lower esters				
1	(Methyl butyrate)	1·27	—	—	6	140	0·47	242·8	24·9
2	30–40	0·05	164·7	—	7	145	0·45	258·8	22·2
3	70–90	0·13	178·0	14·4	8	150	0·74	267·0	21·3
4	90–120	0·15	193·9	15·0	9	153	1·25	270·6	21·1
5	120–130	0·20	222·3	20·8	10	157	1·11	275·4	29·0
						Total	5·82		
					Solid esters				
11	147	0·26	250·9	2·5	18	154	2·86	279·8	4·4
12	150	0·74	262·4	0·8	19	160	1·81	282·0	6·3
13	152	1·79	269·0	1·0	20	165	3·32	294·2	9·9
14	152	3·50	271·7	2·0	21	167	4·06	298·0	11·1
15	153	3·15	275·6	3·0	22	168	3·55	300·4	11·8
16	153	4·25	275·2	2·1	23	Residue	3·08	303·7	14·9
17	154	3·86	277·3	3·5		Total	36·23		
					Liquid esters				
24	140	0·33	243·6	40·9	33	170	5·17	294·0	86·1
25	150	0·46	260·0	49·2	34	175	1·30	293·0	87·5
26	160	0·96	274·6	61·6	35	180	2·47	294·3	86·9
27	164	1·49	288·3	79·3	36	180	7·16	296·2	87·4
28	166	1·59	291·6	83·0	37	180	8·12	296·8	89·2
29	170	2·08	291·1	83·2	38	182	7·63	297·8	90·1
30	170	1·46	294·2	84·0	39	182	4·95	299·0	91·5
31	170	2·47	294·7	85·3	40	Residue	6·20	306·0	91·9
32	170	4·11	294·4	85·9		Total	57·95		

Table III. *Cow no. 1, the composition of the lower, liquid and solid fractions before and during inanition*

Fraction	Methyl esters of the normal fat secreted before inanition				Methyl esters of the fat secreted on the 11th and 12th days of inanition			
	Lower	Liquid	Solid	Total	Lower	Liquid	Solid	Total
Saturated:								
C_4	3·88	—	—	3·88	1·27	—	—	1·27
C_6	0·64	—	—	0·64	—	—	—	—
C_8	0·98	—	—	0·98	0·11	—	—	0·11
C_{10}	1·85	—	—	1·85	0·18	—	—	0·18
C_{12}	2·51	0·05	—	2·56	0·14	—	—	0·14
C_{14}	8·07	3·08	0·74	11·89	1·00	1·25	0·53	2·78
C_{16}	5·32	0·31	17·83	23·46	1·96	0·23	17·84	20·03
C_{18}	—	0·15	11·38	11·53	—	0·04	14·27	14·31
C_{20}	—	—	1·08	1·08	—	—	0·89	0·89
Unsaturated:								
C_{10}	0·24	—	—	0·24	—	—	—	—
C_{12}	0·24	—	—	0·24	—	—	—	—
C_{14}	0·76	0·18	—	0·94	0·19	0·25	—	0·44
C_{16}	0·71	2·44	—	3·15	0·28	1·17	—	1·45
Oleic	—	32·16	3·41	35·57	0·69	49·23	2·70	52·62
Linoleic	—	1·17	—	1·17	—	2·47	—	2·47
C_{20}	—	0·82	—	0·82	—	3·31	—	3·31
Total	25·20	40·36	34·44	100·00	5·82	57·95	36·23	100·00

Table IV. *The component fatty acids of the different butter fats expressed in weight and molar percentages*

	Wt. per 100 g.			Molecules per 100 molecules		
	Cow no. 1		Cow no. 2	Cow no. 1		Cow no. 2
Acid	Before inanition	During inanition*	During inanition†	Before inanition	During inanition*	During inanition†
Saturated:						
C_4	3·5	1·2	2·7	9·7	3·5	7·9
C_6	0·6	—	0·1	1·2	—	0·1
C_8	1·0	0·1	0·1	1·6	0·2	0·2
C_{10}	1·8	0·2	1·0	2·5	0·3	1·5
C_{12}	2·5	0·1	0·6	3·0	0·2	0·7
C_{14}	11·9	2·8	3·8	12·5	3·2	4·3
C_{16}	23·5	20·0	22·1	22·1	20·9	22·1
C_{18}	11·6	14·3	9·9	9·8	13·5	8·9
C_{20}	1·1	0·9	0·9	0·8	0·8	0·8
Total	57·5	39·6	41·2	63·2	42·6	46·5
Unsaturated:						
C_{10}	0·2	—	0·2‡	0·3	—	0·2
C_{12}	0·2	—	0·2‡	0·3	—	0·3
C_{14}	0·9	0·4‡	0·4‡	1·0	0·5	0·5
C_{16}	3·2	1·4	2·0	3·0	1·5	2·0
Oleic	35·9	52·8	51·7	30·5	50·1	46·9
Linoleic	1·2	2·5	0·8	1·0	2·4	0·7
C_{20}	0·8	3·3	3·5	0·6	2·9	2·9
Total	42·4	60·4	58·8	36·7	57·4	53·5
Total of all the acids up to C_{14}	22·6	4·8	9·1	32·1	7·9	15·7

* A sample obtained by mixing the fat secreted on the 11th and 12th days of inanition.
† A pooled sample from the fat secreted on the last 6 days of inanition.
‡ Where the amount of lower acids present is very low and oleic acid predominates as in the fats of inanition, it is probable that the figures marked ‡ are somewhat higher than the true values.

normal and "fasting" fat are arranged in Table III, while the final figures for all three fats are recorded in Table IV.

Linoleic acid. As it was at first thought that inanition might cause marked changes in the amount of linoleic acid present in the different fats, its presence was sought by oxidation methods in various fractions, but in no case could tetrahydroxystearic acid be isolated. This was doubtless due to the fact suggested by the final results of the fractionation that the linoleic acid content of the milk fat from these particular cows was as low as 1–2 % and that the amount of fat available was limited. But as the presence of octadecadienoic acid in normal butter fat has been shown beyond dispute by Green & Hilditch [1935], there can be little doubt that it also existed in smaller amounts in the present case.

Arachidonic acid. The presence of small amounts of arachidonic or some such highly unsaturated acid in the milk fat both before and during inanition was shown by brominating both the last fraction and the residue in the distillation of the liquid esters. The amount of bromo-derivative (Br 65·5 %, M.P. 228° with decomposition), obtained in both cases was less than 0·1 % of the whole.

DISCUSSION

The yields of fat. For all three animals the period of inanition was characterized by a very considerable decrease in the daily secretion of milk fat. Thus it can readily be seen from Fig. 1 that for the first cow, in which inanition was not

complicated by other factors, the yield fell quickly from 568 g. before the fast to 353 g. on the 3rd day, and then more slowly to a mean of 227 g., or 40 % of the original during the last 3 days of the fast. On re-alimentation a further slight drop was noticeable, but 5 days later the daily yield began to increase and at the end of about 6 weeks it had returned to some 60 % of the pre-fast value. With Cow no. 2, in which milk fever symptoms developed on the 3rd day, the secretion of fat and milk almost ceased while this complication lasted, but by the 5th day, the fat production had risen to 12 %, and before the end of the fast to 20 % of the original value. On re-alimentation the yield followed a course almost parallel with that of Cow no. 1 during the same period.

The composition of the fats. Reference to Fig. 2 shows clearly that inanition caused a marked progressive change in the nature of the fats secreted by each of the three cows as judged by their iodine and Reichert Meissl values, and that these changes were similar for all three animals. It will be observed that the initial iodine values of 36·6 and 37·1 for Cows 1 and 2 rose quickly to 46·1 and 47·3 on the 1st day of the fast, and then more slowly to 52·5 and 54·9 by the 12th day, the figures for Cow no. 3 following a similar course till the 7th day when this particular animal was withdrawn from the experiment. Then again the Reichert Meissl values for Cows 1 and 2 fell rapidly in the first 2 days from 26·0 and 33·3 to 14·5 and 22·9 and then still further to 9·8 and 13·8 by the end of the fast. On re-alimentation a slight tendency to return to normal was observed in both values after the first few days and this tendency continued somewhat spasmodically for the next few weeks until figures similar to the pre-fast values were attained. These results suggest that inanition causes a considerable increase in the content of unsaturated acids and a marked fall in that of lower fatty acids normally present as glycerides in butter fat. This was confirmed by more detailed analyses based on fractional distillation methods, for reference to the totals recorded in Tables I and II shows that while the proportion of higher solid esters remained almost unaffected as a result of inanition, the lower esters were reduced from a pre-fast value of 25·2 to 5·8 % during inanition, and that the corresponding figures for the liquid esters showed an almost equal increase from 40·4 to 58 %. Again it will be observed from the weight percentages in Table IV that the sum of all the acids up to and including C_{14} amounted to 22·6 % before the fast and to no more than 4·8 % during inanition, a decrease of 17·8 units, while the oleic acid content rose by 16·9 units from 35·9 to 52·8 %. Calculated also on a molar basis, as shown in Table IV, the fall of 24·2 molecules % in the lower acids is mainly accounted for by a rise of almost 20 molecules % in the oleic acid. It is therefore evident that the chief effect of inanition is the replacement of about 80 % of the lower constituents of the fat mainly by oleic acid, and that the whole of this decrease is distributed over all the lower members from C_4 to C_{14}, although the first and last of these appear to suffer least. Thus the butyric acid content only fell from 3·5 to 1·2 % a trifle more than a third of the original, the myristic by a somewhat similar amount and the others to less than an eighth of their pre-fast values. Of the other saturated acids, the amount of palmitic fell slightly from 23·5 to 20·0 %, while the stearic increased from 11·6 to 14·3 %. That the latter rise had probably some significance is shown best by reference to the molar percentages, where it will be observed that stearic acid increased from 9·8 to 13·5 %, almost a 40 % increase, whereas the fall in palmitic acid only amounted to some 5 % of the pre-fast value. In fact the constancy of the palmitic acid in all three sets of results is worthy of particular notice. The saturated acids represented as C_{20} also remained virtually unchanged. In the unsaturated acids higher than C_{14} and other than oleic, the chief change appeared to be a decrease

in palmitoleic acid from 3·2 to 1·4 % and a most marked rise from 0·8 to 3·3 % in higher unsaturated components represented as "C_{20}".

The results for the fat secreted by Cow no. 2 during inanition are also recorded in Table IV, where it will be seen that with the probable exception of stearic and linoleic acids, the general effect of the fast was the same for both animals.

The relationship between the lipoids of the blood and milk. In discussing the relationship between the blood lipoids and the milk fat it must be remembered that at present there is no means of analysing the triglyceride fatty acids of the blood by themselves but only when mixed with those originally present as cholesteryl esters. There is therefore every possibility that the triglyceride acids of the blood may change and yet the change may be impossible to detect, and as these acids probably form one of the most important sources of milk fat, the disadvantage inherent in this fact for research work of the present type cannot be overestimated. At the same time, bearing this limitation in mind, it is well worth observing the almost complete absence of change already noted in the blood fatty acids and particularly in those of the non-phosphatide fraction of the plasma during inanition as compared with the very striking alterations brought about in the fat of the milk. In fact, the latter suggest that the normal equilibrium existing between the amount of fat actually formed in the gland and that arising directly from the blood was very considerably disturbed. For example, the enhanced concentration of higher unsaturated compounds of the C_{20} type during the fast might well be taken as indicating that there was an increase in the unsaturated acids arising directly from the blood in which the proportion of these acids has been shown by Parry & Smith [1936] to be exceedingly high, and that the proportion of fat synthesis in the gland was considerably decreased.

The greatest change, however, was in the amount of lower fatty acids present and these are worthy of special consideration. With regard to their source in normal butter fat there are probably three main possibilities. In the first place, they may pre-exist in the blood and arise from it directly. Secondly, they may be formed in the mammary gland itself by breakdown of oleo-glycerides as suggested by Hilditch & Thompson [1936] and by Hilditch & Paul [1936]. Thirdly, they may arise, again in the gland itself, as by-products in the synthesis of the fat from some form of carbohydrate material. The theory that they may arise directly from the blood gains support from the suggestion that lower fatty acids may be formed as by-products of rumination in the cow and that these may then be transported by the blood to the mammary gland. In human milk the content of these lower constituents is exceedingly small. From the present work this theory might also appear to receive confirmation from the fact that when rumination ceased as a result of the enforced fast, the lower fatty acid content of the milk also fell. In this connexion two points should, however, be noted. In the first place it would be natural for these lower compounds, if they exist in blood, to be present in the non-phosphatide fraction of the plasma. But the results recorded in Table II of the previous paper (p. 1861) suggest that if any of these lower acids were present as esters in this fraction, their amount must have been very small, and that they certainly did not appear to decrease during inanition. It is always possible, however, that during the analysis of the blood lipoids free water-soluble acids were lost, for such an occurrence would be exceedingly difficult either to detect or avoid. In the second place, if the reduction in these lower constituents of milk fat were due in the present instance to a deficiency of the same acids in the blood, it would be natural to expect that their decrease in the milk fat during inanition would be counterbalanced by increases fairly evenly distributed over the other constituents, whereas it has already been

shown that almost the whole loss is made good by oleic acid alone. Indeed this last observation might well be taken as evidence in support of Hilditch's suggestion that the lower acids arise by the oxidation of oleo-glycerides in the gland itself, for if this were so, the catabolism of these glycerides might not be so essential during inanition when the general activity of the gland was considerably reduced, with the consequence that the content of the lower constituents would fall and that of oleic acid rise. Recent work, however, by Graham *et al.* [1938], based partly on respiratory quotient measurements, has definitely suggested that fat metabolism in the mammary gland probably takes the form of synthesis rather than of breakdown. The explanation of the present results may therefore be that the lower compounds are formed as by-products in the synthesis of oleic acid, but that during inanition, when the gland is producing much less than its usual amount of fat, the smaller amount of synthesis taking place is able to proceed to its final stages, so that the lower acids are almost completely converted into oleic. This suggestion gains support from the well-known fact that diets rich in carbohydrates and poor in fat cause an increase in the lower fatty acids of the milk fat, while those poor in carbohydrates and rich in fat have the reverse effect, indicating that with diets on which fat synthesis would probably be most necessary the lower fatty acids increase. At present it is impossible to decide as to which of these various theories is correct but it is hoped that future work now contemplated will throw more light on this particular aspect of the subject. At present the fact of chief importance emerging from the results just described is the inverse relationship existing between the oleic acid on the one hand and the lower acids on the other.

SUMMARY

1. Although in the previous paper no appreciable change was observed during inanition in the nature of the blood lipoids of three lactating cows, very considerable changes have been found in the milk fat, and these have been studied in detail.

2. The chief change was a decrease of about 80 % in the original content of lower acids up to and including C_{14}, a deficiency which was almost entirely made good by an increase in the content of oleic acid.

3. The fact that oleic acid tended so completely to replace the lower constituents has been discussed in connexion with different theories previously put forward to explain the occurrence of these lower homologues in milk.

4. A description is given of a modified procedure for the analysis of butter fat by the fractional distillation method from which prolonged distillation of the saponified fat in steam is omitted.

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CCXLII. ENZYMIC PROTEOLYSIS

I. LIBERATION OF AMMONIA FROM PROTEINS[1]

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THE isolation of asparagine [Damodaran, 1932] and of glutamine [Damodaran *et al.* 1932] from enzymic digests of proteins has given direct demonstration of the existence of amide groups in the protein molecule. Before, however, any attempt can be made to decide if the ammonia, which is an invariable product of protein hydrolysis, arises solely from dicarboxylic acid amides, it becomes essential to have more exact information than is at present available on the deamidizing activity of the proteolytic enzymes. Such information is in the first place necessary to enable a proper choice of enzymes and conditions of experiment to be made, so that digestion of protein could be effected with the least amount of decomposition of the ammonia-yielding complexes. Secondly, comparative study of the liberation of ammonia from proteins and from synthetic substrates is likely to provide analogies from which the nature of the precursors of ammonia might be inferred.

The rate of liberation of ammonia in tryptic digests of casein, gliadin and Witte's peptone was studied by Hunter & Smith [1925], who found that the
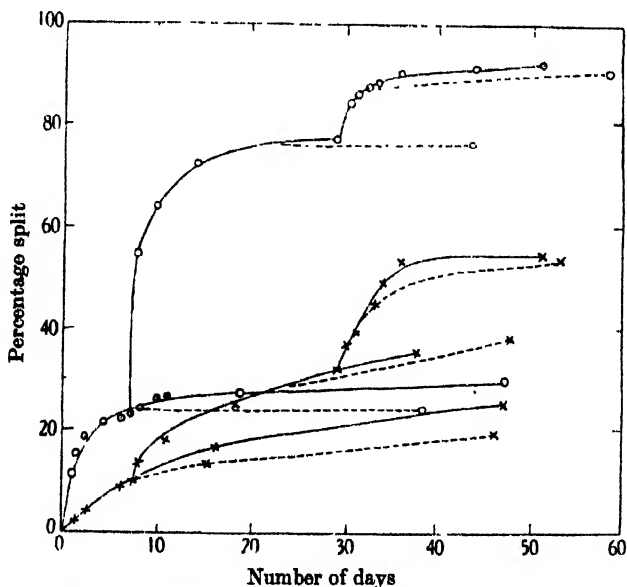
Fig. 1. Digestion of casein. ⊙ Amino. × Amide. — Digest. ---- Control.

formation of ammonia proceeded much more slowly than the liberation of amino groups. According to these authors "the differences were so great as to suggest

[1] Read before the Indian Science Congress, January 1938.

that the two processes are really catalysed by different enzymes and that pure
trypsin might produce from proteins no ammonia at all".

As a preliminary to the work already cited on the isolation of asparagine
and glutamine, a number of experiments were carried out on the splitting of
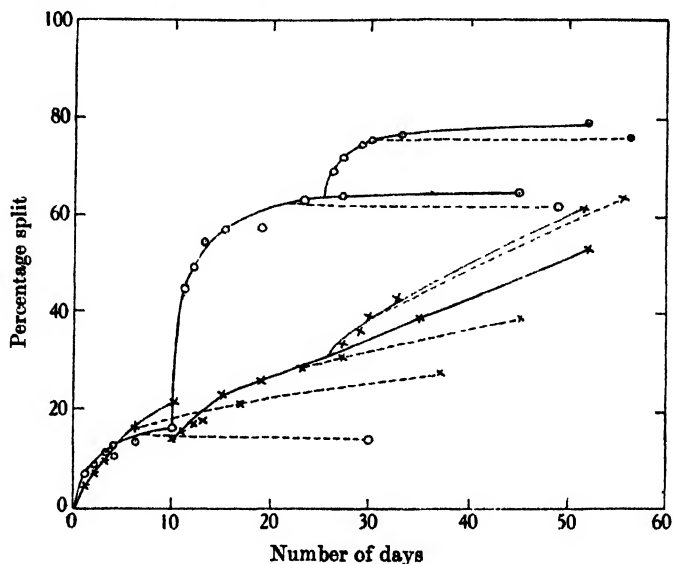
Fig. 2. Digestion of gliadin. ⊙ Amino. × Amide. — Digest. ---- Control.
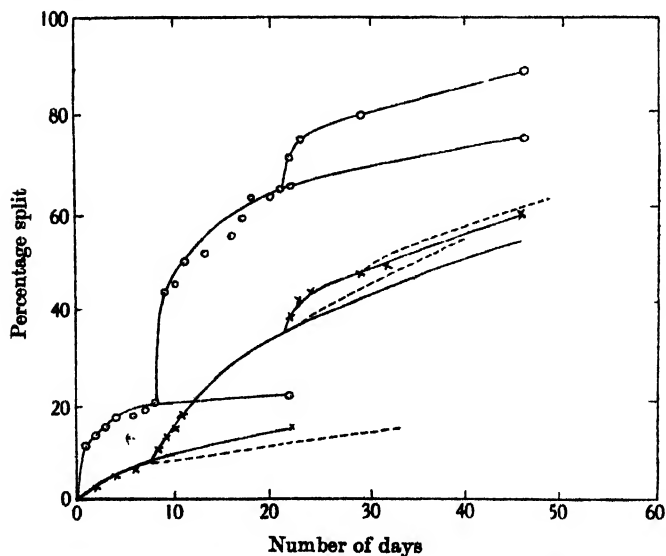
Fig. 3. Digestion of edestin. ⊙ Amino. × Amide. — Digest. ---- Control.

amide and peptide bonds during the action of proteolytic enzymes on gliadin
and edestin. Attention was, however, mainly directed to securing the conditions
under which a far-reaching hydrolysis of protein could be effected with minimal

liberation of ammonia and no conclusions were drawn as to any relation between proteolytic and deamidizing actions of the enzymes used.

The present paper describes a systematic study of the formation of ammonia and the hydrolysis of peptide linkages during the action of pepsin, trypsin, erepsin and papain on three typical proteins, casein, edestin and gliadin. Figs. 1–3 represent graphically the whole course of the digestion of each of these proteins by the three animal proteases acting in succession. The numerical data and details of procedure are given in Tables I, II, III. It will be seen from the curves, which show the progressive liberation of amino groups and of free ammonia during the course of digestion, that the rates of splitting in the two cases do not by any means run parallel. With each enzyme, in the earlier stages of action

Table I. *Pepsin, trypsin and erepsin on casein*

A. 22 g. casein (N 15·68 %) dissolved in about 500 ml. $N/10$ HCl + 475 ml. water + 1 g. B.D.H. pepsin dissolved in 25 ml. water.

B. 60 ml. of A (at the end of 8 days) boiled at pH 6 to inactivate pepsin, brought back to pH 1·6 and made up to 100 ml.

C. 450 ml. of A (at the end of 7 days) boiled at pH 6, brought to pH 8·6 with NaOH, 25 ml. of a solution of 0·5 g. of B.D.H. trypsin and sufficient water to make up to 500 ml.

D. 30 ml. of C (at the end of 15 days on trypsin) boiled at pH 6, adjusted to pH 8·6 and made up to 50 ml.

E. 140 ml. of C (after 22 days on trypsin) inactivated at pH 6, brought to pH 7·6 and made up to 250 ml. after the addition of 15 ml. of erepsin solution. After inactivation and before addition of erepsin the digest had to be left in the ice-chest for about 3 weeks.

F. 30 ml. of E boiled at pH 6 and made up to 50 ml. after adjusting to pH 7·6.

	Hydrolysis %											
	Pepsin				Trypsin				Erepsin			
	A		B (after inactivation)		C		D (after inactivation)		E		F (after inactivation)	
Time hr.	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide
0	—	—	—	—	27·30	8·50	—	—	79·70	35·00	—	—
1	33·29	—	—	—	—	—	—	—	—	—	—	—
2	3·43	—	—	—	39·10	8·50	—	—	80·80	35·00	—	—
4	6·57	—	—	—	46·10	—	—	—	—	—	—	—
6	9·43	—	—	—	—	—	—	—	81·30	35·00	—	—
8	10·42	—	—	—	54·05	—	—	—	—	—	—	—
12	11·00	0·80	—	—	55·60	—	—	—	—	—	—	—
days												
1	15·29	1·60	—	—	62·10	10·10	—	—	84·60	37·20	—	—
2	18·43	3·06	—	—	63·60	11·70	—	—	85·80	39·70	—	—
3	—	—	—	—	—	—	—	—	86·40	43·60	—	—
4	21·00	4·95	—	—	67·10	14·30	—	—	87·30	44·20	—	—
5	—	—	—	—	70·30	16·90	—	—	88·90	49·30	—	—
6	21·86	6·82	—	—	—	—	—	—	—	—	—	—
7	23·30	8·29	—	—	—	—	—	—	90·70	53·60	90·80	53·10
8	24·10	9·49	24·25	8·62	—	—	—	—	—	—	—	—
10	25·90	10·56	—	—	—	—	—	—	—	—	—	—
11	26·40	11·23	—	—	73·10	24·10	—	—	—	—	—	—
15	—	—	—	—	76·30	29·30	76·70	29·70	—	—	—	—
16	27·57	16·31	24·25	12·82	—	—	—	—	—	—	—	—
22	—	—	—	—	77·90	33·10	—	—	92·90	57·40	90·80	57·60
31	—	—	—	—	—	—	76·70	37·60	—	—	—	—
47	30·13	25·00	24·25	18·84	—	—	—	—	—	—	—	—
49	—	—	—	—	—	—	76·70	45·30	—	—	—	—
66	—	—	—	—	—	—	76·70	52·20	—	—	—	—

Table II. *Pepsin, trypsin and erepsin on gliadin*

A. 11 g. gliadin (N 17·25 %) suspended in about 400 ml. of $N/20$ HCl and treated with 0·5 g. of pepsi dissolved in a few ml. of $N/20$ HCl; clear solution obtained after incubation for 3 hr.

B. 30 ml. of A boiled at pH 6 on the 7th day, pH brought again to 1·6 and made up to 50 ml.

C. 350 ml. of A on the 10th day boiled at pH 6, brought to pH 8·6, 0·4 g. B.D.H. trypsin added and mad up to 500 ml.

D. 30 ml. of C (after 20 days on trypsin) boiled at pH 6, reaction adjusted to pH 8·6 and made up to 50 ml.

E. 175 ml. of C (after 16 days on trypsin) boiled at pH 6, adjusted to pH 7·8 and 25 ml. of erepsin solution added.

F. 30 ml. of E boiled at pH 6 after 3 days on erepsin, pH readjusted to 7·8 and made up to 50 ml.

Hydrolysis %

| | Pepsin | | | | Trypsin | | | | Erepsin | | | |
| | A | | B (after inactivation) | | C | | D (after inactivation) | | E | | F (after inactivation) | |
Time hr.	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide
0	—	—	—	—	16·40	15·12	—	—	63·50	29·00	—	—
1	—	—	—	—	23·70	15·12	—	—	—	—	—	—
2	—	—	—	—	30·16	15·12	—	—	65·50	29·00	—	—
4	—	—	—	—	35·60	15·12	—	—	66·10	29·00	—	—
6	—	—	—	—	38·30	15·12	—	—	67·30	29·00	—	—
7	0·81	0·04	—	—	—	—	—	—	—	—	—	—
10	2·24	0·90	—	—	—	—	—	—	—	—	—	—
12	—	—	—	—	43·10	15·29	—	—	67·50	29·90	—	—
days												
1	6·52	4·05	—	—	44·50	15·70	—	—	69·20	30·50	—	—
2	8·56	7·70	—	—	49·00	16·29	—	—	72·00	33·20	—	—
3	10·52	10·68	—	—	54·30	17·64	—	—	—	—	73·30	39·20
4	12·16	12·69	—	—	—	—	—	—	74·40	36·50	—	—
5	—	—	—	—	56·70	23·18	—	—	75·50	39·20	—	—
6	13·72	16·38	—	—	—	—	—	—	—	—	—	—
7	—	—	14·10	16·55	—	—	—	—	—	—	—	—
8	—	—	—	—	—	—	—	—	76·10	43·10	—	—
9	—	—	—	—	57·70	25·20	—	—	—	—	—	—
10	16·54	21·60	—	—	—	—	—	—	—	—	—	—
11	—	—	—	—	—	—	—	—	—	—	73·30	48·00
13	—	—	—	—	63·60	28·40	—	—	76·50	45·60	—	—
17	—	—	14·10	20·30	64·00	30·41	—	—	—	—	—	—
20	—	—	—	—	—	—	64·60	28·34	—	—	—	—
27	—	—	—	—	—	—	—	—	78·80	60·80	—	—
32	—	—	—	—	—	—	64·60	38·59	—	—	73·30	60·70
49	—	—	—	—	—	—	64·60	54·04	—	—	—	—

when the rate of peptide hydrolysis is at its highest, ammonia liberation is hardly noticeable and becomes significant only after the products of digestion have accumulated to some extent. On the other hand when the most active stage of proteolysis is past and formaldehyde titrations are increasing only very slowly, as indicated by the flattening of the amino-N curves, the liberation of ammonia continues at a steady rate and shows no tendency to slow down. These results are clearly brought out in Table IV and Figs. 4 and 5, in which the extents of amide and peptide hydrolysis at intervals at the beginning and end of the digestion are compared. The observations are readily explicable on the assumption that the ammonia formed is, at least in great part, not directly connected with

Table III. *Pepsin, trypsin and erepsin on edestin*

A. 17·5 g. of crystalline edestin stirred up with 375 ml. of $N/10$ HCl and kept at 37° for 1 hr., 350 ml. of water and 0·75 g. of pepsin in 25 ml. added to the clear solution.

B. 30 ml. of A boiled at pH 6 on the 18th day brought to pH 1·6 and made up to 50 ml.

C. 425 ml. of A on the 9th day boiled at pH 6 brought to pH 8·6, 0·5 g. B.D.H. trypsin added and made up to 500 ml.

D. 30 ml. of C boiled at pH 6 (after 15 days on trypsin) adjusted to pH 8·6 and made up to 50 ml.

E. 175 ml. of C (after 13 days on trypsin) boiled at pH 6 adjusted to pH 7·8 and 25 ml. of erepsin solution added.

F. 40 ml. of E boiled at pH 6 (after 17 days on erepsin), readjusted to pH 7·8 and made up to 50 ml.

Hydrolysis %

| | Pepsin | | | | Trypsin | | | | Erepsin | | | |
| | A | | B (after inactivation) | | C | | D (after inactivation) | | E | | F (after inactivation) | |
Time hr.	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide
0	—	—	—	·	20·10	11·50	—	··	62·10	34·80	—	—
1	—	—	·	—	27·10	—	—	—	—	—	—	—
2	2·44	···	—	···	—	···	—	··	65·10	34·80	—	—
3	5·49	—	·	—	31·80	11·50	··	—	—	—	—	—
4	—	·	—	···	—	—	—	—	65·50	—	—	—
5	—	···	—	·	33·40	···	—	—	—	—	—	—
6	7·93	—	··	—	—	—	—	—	66·40	—	—	—
8	—	···	—	·	37·80	11·80	—	—	—	—	—	—
12	10·54	0·34	—	—	···	—	—	—	—	—	—	—
days												
1	11·10	1·02	—	—	42·30	13·40	—	—	73·20	37·10	···	—
2	13·29	2·38	···	—	44·60	15·40	—	—	76·00	40·70	—	—
3	14·76	4·14	···	—	49·70	17·30	—	—	76·20	42·60	—	—
4	16·83	5·21	—	—	—	—	—	—	76·80	—	—	—
5	—	···	—	··	53·40	21·40	—	—	—	—	—	—
6	17·68	6·18	—	—	··	—	—	—	—	—	—	—
7	18·29	7·35	—	—	—	···	—	—	—	—	—	—
8	—	—	—	···	57·80	27·70	—	—	79·20	46·30	79·80	43·60
9	—	—	—	—	59·90	29·00	—	—	—	—	—	—
10	···	8·22	—	—	62·00	29·90	··	—	—	···	—	—
12	—	—	—	—	62·30	31·00	—	—	··	—	—	—
14	—	—	—	···	65·30	35·40	—	—	—	—	—	—
15	—	—	—	—	—	—	63·20	35·90	—	—	—	—
17	—	—	—	—	—	···	—	—	—	—	80·50	56·60
18	—	—	17·48	7·19	—	—	—	—	—	—	—	—
22	21·96	14·08	—	—	—	—	—	—	—	—	—	—
24	···	—	—	—	—	—	63·20	48·10	—	—	—	—
25	···	—	—	—	—	—	—	—	88·90	59·20	—	—
32	—	—	18·90	13·98	···	—	—	—	—	—	—	—
38	—	—	—	—	74·00	54·70	···	—	—	—	—	—
42	—	—	—	—	—	—	—	—	89·10	67·50	—	—
43	—	—	—	—	—	—	—	—	—	—	81·20	61·60
56	—	—	18·91	22·77	—	—	63·20	63·90	—	—	—	—
74	—	—	18·91	22·77	—	—	—	—	—	—	—	—

proteolytic action but arises from the decomposition of the primary products of protein cleavage similar to the γ-peptides of glutamine studied by Melville [1935]. It also seems safe to conclude that this secondary decomposition is not enzymic in nature but is brought about by the acidic or alkaline reactions involved. Thus when peptic, tryptic and ereptic digests, in which the increase in amino groups have progressed considerably, are boiled to inactivate the enzyme

and incubated the formation of ammonia goes on at approximately the same rate as in unboiled digests containing the active enzyme, although the former
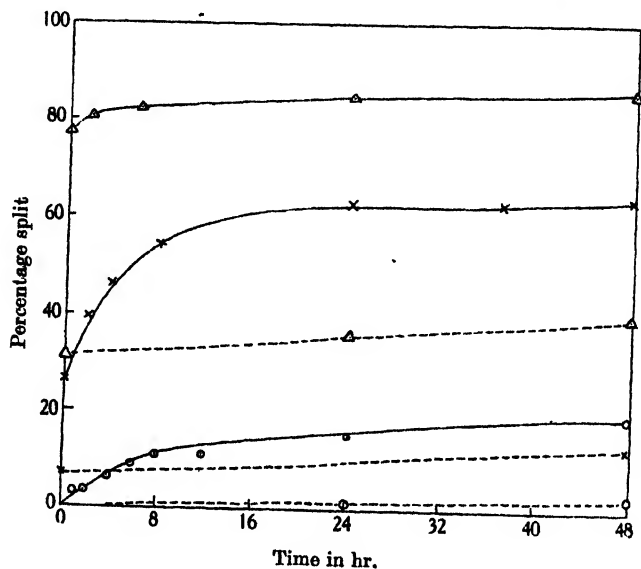
Fig. 4. Digestion of casein, initial stages. — Amino. · · · Amide.
⊙ Pepsin. × Trypsin. △ Erepsin.
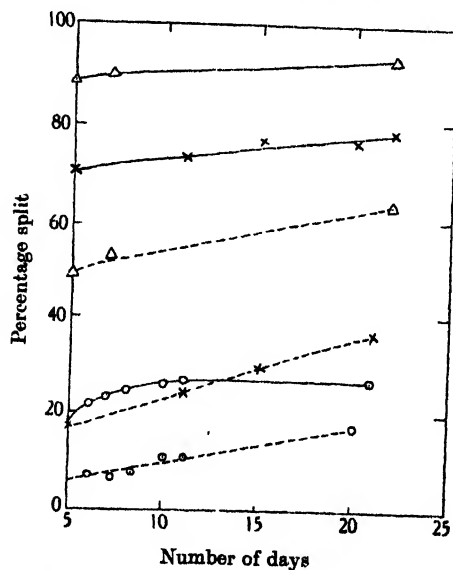
Fig. 5. Digestion of casein, final stages. ⊙ Pepsin. × Trypsin.
△ Erepsin. · · · · Amide. — Amino.

show no further increase in amino groups. Similar results were obtained with purified trypsin and erepsin (Fig. 6 and Table V).

Table IV

Protein	Enzyme	Interval in days		% hydrolysis in the interval	
		From	To	Peptide	Amide
Casein	(a) Pepsin	0	2	18·43	3·06
		8	47	6·03	15·51
	(b) Control	8	47	0·00	10·22
	(a) Trypsin	0	1	34·80	1·60
		15	22	1·60	3·80
	(b) Control	15	66	0·00	22·50
	(a) Erepsin	0	1	4·90	2·20
		7	22	2·20	3·80
	(b) Control	7	22	0·00	4·50
Gliadin	(a) Pepsin	0	2	8·56	7·70
		6	10	2·82	5·22
	(b) Control	6	36	0·00	10·44
	(a) Trypsin	0	2	32·60	1·17
		17	35	0·80	17·59
	(b) Control	17	46	0·00	25·74
	(a) Erepsin	0	2	8·50	4·20
		2	27	6·80	27·60
	(b) Control	3	31	0·00	21·50
Edestin	(a) Pepsin	0	2	13·29	2·38
		22	64	7·29	10·73
	(b) Control	18	74	1·33	15·58
	(a) Trypsin	0	2	24·50	1·90
		14	38	8·70	19·30
	(b) Control	15	56	0·00	28·00
	(a) Erepsin	0	2	13·90	5·90
		8	42	9·90	21·20
	(b) Control	8	43	1·40	17·50

Table VI gives the results of an interesting experiment in which the deamidizing action of pepsin was tested on casein previously digested with trypsin and erepsin. It is seen that pepsin is unable to bring about any further hydrolysis of peptide groups but that ammonia production takes place as in a peptic digest of native casein. Further, the amount of ammonia produced is not higher than in a portion of the trypsin-erepsin digest kept at the pH necessary for the action of pepsin, but without the addition of the latter. This experiment brings out another interesting result not, however, connected with deamidation. When casein is acted upon by pepsin, trypsin and erepsin in that order the extent of hydrolysis is nearly 90 % of complete hydrolysis (about 20 % due to pepsin and the remaining 70 % due to the other two enzymes). When trypsin and erepsin are used first the splitting produced by them is still 70 %, but pepsin is now unable to act further on this digest and contribute its quota to bring up the hydrolysis to the usual 90 %. This result would suggest that the deciding factor for the action of pepsin is the position of the bonds attacked rather than their nature.

Table VII and Fig. 8 show the action of papain on casein, and Table VIII the action of papain on edestin. The results summarized in Table IX for the action of papain on casein are entirely different from those obtained with the animal proteases. It must be stated at the outset that, notwithstanding the

Table V. *Purified trypsin and erepsin on casein*

A. 15 g. casein dissolved in very dilute alkali, boiled for a minute and made up to 700 ml. at pH 8·6, 100 ml. of erepsin-free trypsin solution, also at pH 8·6, added.

B. 200 ml. of A on the 26th day boiled at pH 6 and after adjusting the reaction to pH 7·8, 25 ml. of trypsin-free erepsin added and the volume made up to 250 ml.

	Hydrolysis %			
	A Erepsin-free trypsin		B Trypsin-free erepsin	
Time hr.	Peptide	Amide	Peptide	Amide
0	—	—	48·70	27·31
1	3·50	—	—	—
2	6·10	—	50·44	—
6	—	—	55·60	27·52
days				
1	32·90	1·76	63·50	31·30
2	35·10	5·18	69·40	34·66
3	—	—	74·80	36·97
4	40·20	8·47	75·20	39·50
5	—	—	76·60	40·55
6	45·10	9·24	—	—
7	—	—	76·80	44·75
8	45·40	11·96	—	—
11	—	—	76·90	50·21
12	45·40	16·59	—	—
24	—	—	79·30	55·88
25	48·80	26·57	—	—
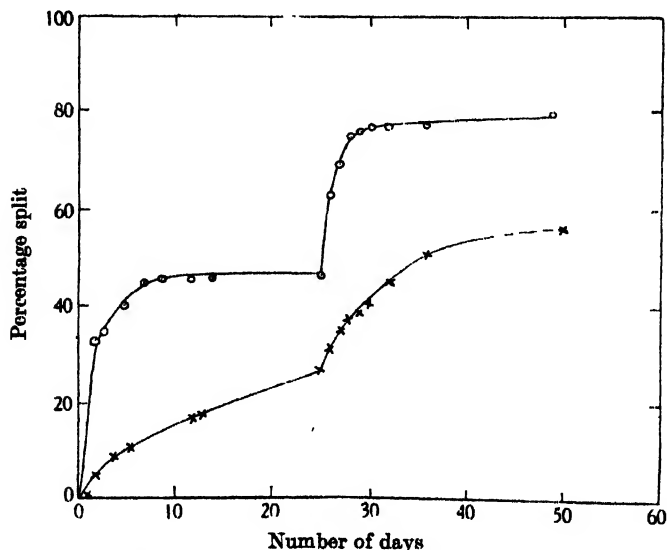
Fig. 6. Purified trypsin and erepsin on casein. ⊙ Amino. × Amide.

statements in the literature that activated papain is able to act on natural proteins, we have never succeeded in getting any of the proteins used in these experiments into solution by the action of papain-HCN either with commercial samples or with the enzyme prepared from the fresh latex. In all experiments

Table VI. *Pepsin after trypsin and erepsin on casein*

A. 22 g. casein dissolved in dilute NaOH, pH adjusted to 8·6, 1 g. B.D.H. trypsin added and made up to 1 l.

B. 800 ml. of A on the 8th day boiled at pH 6, made up to 1 l. at pH 7·8, with 100 ml. of erepsin solution and water.

C. 400 ml. of B on the 9th day inactivated by boiling at pH 6 brought to pH 1·8 with N HCl and made up to 500 ml. 250 ml. pipetted out and 0·25 g. of B.D.H. pepsin powder stirred in.

D. 250 ml. of C without the addition of pepsin.

| | Hydrolysis % | | | | | | | |
| | Trypsin A | | Erepsin B | | Pepsin C | | Pepsin D | |
Time hr.	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide
0	—	—	46·38	14·25	72·90	30·08	—	—
1	14·10	—	—	—	72·90	30·31	—	—
2	—	—	49·19	—	72·90	30·31	—	—
4	—	—	50·66	14·25	72·90	31·09	—	—
6	—	—	—	—	72·90	31·20	—	—
7	25·10	0·27	—	—	—	—	—	—
days								
1	35·60	0·95	59·57	16·65	72·90	35·75	72·90	35·88
2	39·20	4·89	62·91	19·27	72·90	41·40	72·90	40·56
3	—	—	—	—	72·90	44·35	72·90	42·04
4	43·70	8·98	70·52	23·27	—	—	—	—
5	45·70	9·93	71·25	—	—	—	—	—
6	—	—	72·56	29·25	—	—	—	—
7	48·20	13·56	—	—	72·90	48·37	72·90	47·46
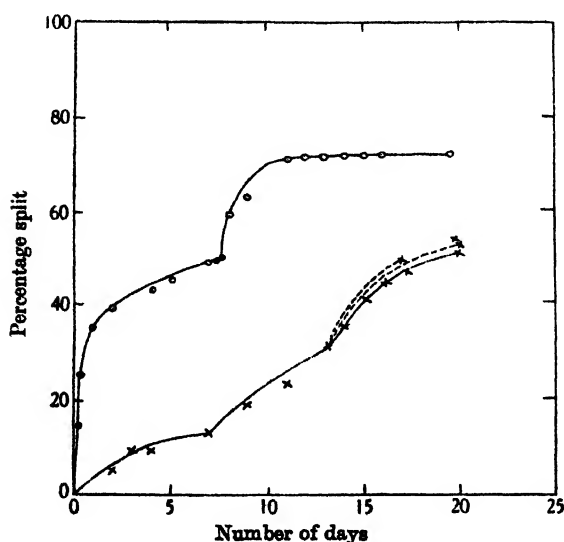
Fig. 7. Pepsin after trypsin and erepsin. ◉ Amino. × Amide.
— Digest. ‑‑‑‑ Control.

Table VII. *Papain and papain-HCN on casein*

Casein digested with pepsin-HCl for 24 hr. and inactivated by boiling at pH 5.

A. 200 ml. of casein peptone and 0·225 g. papain powder in 50 ml. of water.

B. 40 ml. of A boiled on the 34th day and made up to 50 ml.

C. 200 ml. of casein peptone and 50 ml. of papain-HCN (obtained by adding 10 ml. of 3 % KCN solution neutralized to methyl red with HCl and incubating at 37° for 1 hr).

D. 80 ml. of C boiled after 15 days and made up to 100 ml.

| | Papain | | | | Papain-HCN | | | |
| | A | | B | | C | | D | |
Time hr.	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide
0	13·55	8·81	—	—	13·55	8·81	—	—
5	—	—	—	—	31·25	18·84	—	—
days								
1	21·43	18·62	—	—	43·78	29·64	—	—
2	23·82	19·15	—	—	47·06	32·66	—	—
4	24·33	21·24	—	—	53·21	36·85	—	—
6	25·00	22·56	—	—	56·36	38·56	—	—
9	—	—	—	—	60·39	41·71	63·81	41·30
10	—	—	—	—	—	—		
15	—	—	—	—	62·63	44·27	—	—
16	31·23	26·89	—	—	—	—	63·81	41·62
17	—	—	—	—	—	—		
34	33·36	28·60	33·40	28·62	68·92	48·93	63·81	44·25
35	—	—	—	—	—	—		
41	—	—	33·40	28·77	—	—		
59	—	—	33·40	30·16	—	—		
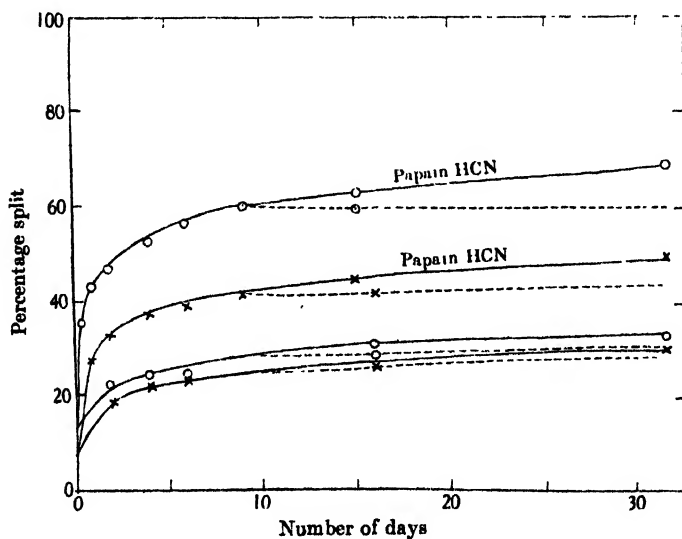
Hydrolysis %

Fig. 8. Casein peptone + papain. ⊙ Amino. × Amide.
— Digest. ---- Boiled control.

Table VIII. *Papain and papain-HCN on edestin*

Edestin digested with pepsin-HCl for 24 hr.
A, C and D prepared from edestin peptone as in Table VIII.

	Hydrolysis %					
	Papain A		Papain-HCN			
			C		D	
Time hr.	Peptide	Amide	Peptide	Amide	Peptide	Amide
0	12·96	0·64	12·96	0·64	—	—
2	—	—	18·68	4·67	—	—
5	—	—	23·40	7·51	—	—
days						
1	19·36	6·05	34·51	13·90	—	—
2	21·80	6·87	41·08	18·05	—	—
5	—	—	47·57	22·22	—	—
9	22·22	8·27	52·36	26·83	52·40	26·90
13	—	—	55·55	28·03	—	—
27	—	—	—	—	52·40	29·00

Table IX

Enzyme, etc.	Interval in days		% hydrolysis in the interval	
	From	To	Peptide	Amide
Casein peptone and papain-HCN	0	1	30·23	20·83
	1	15	18·85	14·63
	15	34	6·29	4·66
Control	9	34	0·00	2·95
Casein peptone and papain	0	1	7·88	9·81
	1	16	9·78	8·27
	16	34	2·15	1·71
Control	9	34	0·00	1·54

with papain, therefore, substrates used were proteins which were brought into solution by a 24 hr. digestion with pepsin. There is no doubt that in papain digests ammonia formation is connected with enzyme action. There is a certain parallelism between amide and peptide hydrolyses and further, activation by HCN accelerates the liberation of both amino groups and of free ammonia. The ammonia formed by secondary effects of the reaction medium in boiled digests is only a small fraction of the total ammonia produced during enzyme·action. Further investigations are, however, necessary before it can be decided whether the power to hydrolyse peptide and amide bonds resides in the same enzyme or whether papain contains two components with those specific functions.

EXPERIMENTAL

Materials. The proteins used were prepared in the laboratory shortly before use. We have invariably found that commercial preparations of proteins are entirely unsuitable for quantitative digestion experiments on account of their physical properties which cause difficulty in getting them into solution or uniform suspension. The preparations were obtained by the usual methods, dehydrated with acetone without allowing prolonged contact, and the acetone removed rapidly by a current of air.

Enzymes. In most of the experiments B.D.H. preparations of pepsin and trypsin were used. In the series with purified enzymes erepsin-free trypsin (proteinase + carboxypolypeptidase) was prepared from defatted pig's pancreas by the method described by Waldschmidt-Leitz & Harteneck [1925]. Erepsin solution was obtained for ordinary experiments from an 87 % glycerol extract of pig's intestinal mucosa by the following procedure, all operations involved being carried out at 0°. One volume of the glycerol extract was diluted with three volumes of ice-cold water and centrifuged. The supernatant was mixed with one-tenth its volume of $N/10$ acetic acid and again centrifuged to remove extraneous protein. The clear centrifugate was brought to pH 7·8 with alkali and immediately used. Trypsin-free erepsin (aminopolypeptidase + dipeptidase) was obtained from the crude solution obtained above by adsorption at pH 4·8 on aluminium hydroxide, C_y, and elution with phosphate buffer at pH 8·3 [Waldschmidt-Leitz & Schaffner, 1926].

Papain used in these investigations was prepared in the laboratory from fresh papaya latex. The latex liberated by scarifying the skin of the fresh green fruits with a bone spatula was collected in a porcelain dish, the thick white fluid squeezed through muslin and precipitated by adding several volumes of ice-cold acetone. The precipitate was washed twice with acetone and dried over sulphuric acid *in vacuo*. A snow-white powder was thus obtained which dispersed in water to give a slightly opalescent solution. The preparation gave tests for peroxidase. For activation by HCN the method of Willstätter & Grassmann [1924] was used.

Preparation of digests. The digests were generally made up to contain 2 g. of protein and 0·1 g. of enzyme per 100 ml.

The starting of the digestion with pepsin was slightly different with the three proteins. With casein a solution in $N/10$ HCl could be readily obtained and after the addition of water and pepsin solution readings could be commenced immediately after mixing. Edestin suspended in $N/10$ HCl went into solution on being kept at 37° for 1 hr. with repeated shaking. Gliadin on the other hand formed a sticky mass on being suspended in $N/10$ HCl and went into solution only after digestion with pepsin for 3 hr. In all cases quantitative measurements were commenced only after homogeneous solutions were obtained. For digestion with papain the proteins were first brought into solution by means of pepsin which was then inactivated by boiling.

At the end of the action of pepsin, after leaving a small quantity for studying the effect of prolonged digestion, the main portion of the digest was brought to pH 6 and boiled to inactivate the enzyme. At this reaction it was found that boiling for 15 min. caused no appreciable increase in amino-N or ammonia. The solution was now cooled to 37° and, after the reaction was adjusted to pH 8·6 with NaOH, transferred to a measuring flask, mixed with a 5 % trypsin solution containing one-twentieth the weight of the protein now present, and the volume made up at 37°. Readings were commenced immediately. A similar procedure was adopted in transferring the digest from trypsin to erepsin, 100 ml. of the latter being added to each 900 ml. of the inactivated tryptic digest. After the action of each enzyme had proceeded to a considerable extent a portion of the digest was inactivated and incubated to study the secondary formation of ammonia from the cleavage products under the influence of the reaction of the medium. The inactivation was brought about by boiling at pH 6, the reaction being then adjusted to the original pH of the particular digest.

Methods of analysis. On account of the difficulty of quantitatively dissolving a weighed amount of protein in a definite volume of liquid, calculation of percentage hydrolysis was made not on the basis of the weight of protein but on

the results of analysis of an aliquot of the digest subjected to complete hydrolysis by HCl and in which amino-N and ammonia-N were determined in the same way as in the enzyme digest. Such analyses were carried out after the addition of each enzyme and also in digests inactivated by boiling.

Nitrogen was determined by micro-Kjeldahl, amino nitrogen by Sørensen's formol titration (from pH 6·8 in aqueous solution to distinct pink with phenolphthalein in formalin). Free ammonia was determined by distillation *in vacuo* at 40° in the apparatus of Parnas & Heller [1924] but without the use of steam; a 10% solution of sodium carbonate was used for making the solution alkaline; it was found that distillation was always complete in 10 min. during which time 9–10 ml. of the distillate were collected.

Certain unavoidable errors in the methods of analysis have to be noted, viz. the interference in the formaldehyde titration by the ammonia present in the digest, and in the case of purified ereptic digests by phosphate in the buffer solution used for elution, as also the possible formation of ammonia from cyanide during vacuum distillation with sodium carbonate of papain-HCN digests. But experimentally and by calculation it could be shown that these errors are not of sufficient magnitude to affect the significance of the results obtained.

SUMMARY

A study has been made of the progressive liberation of ammonia and of amino-groups during the action of pepsin, trypsin, erepsin and of papain on three typical proteins, casein, edestin and gliadin.

It is shown that amide and peptide hydrolyses do not by any means run parallel and that the ammonia formed is, at least in great part, not directly connected with proteolytic action but arises from the decomposition of the primary products of protein cleavage. This secondary decomposition is not enzymic in nature but is brought about by the acidic or alkaline reactions of the digestion media.

In papain digestion, on the other hand, ammonia formation appears to be an enzymic function. Papain either possesses deamidase activity itself or contains a component which has such activity.

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CCXLIII. THE PREPARATION OF CONCEN-
TRATED ANTIGONADOTROPIC
FACTOR (ANTIPROLAN)

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THOMPSON [1937] showed that when antigonadotropic sera are salted out with 33–50 % sat. $(NH_4)_2SO_4$, the antigonadotropic factor may be quantitatively recovered from the pseudoglobulin fraction. Zondek & Sulman [1937] described independently several methods of preparation of the antigonadotropic factor, viz. precipitation with acetone and salting out with $(NH_4)_2SO_4$ directly from the serum or from a solution of an acetone-powder prepared therefrom. Harington & Rowlands [1937] confirmed and extended these results in an extensive series of experiments.

The following methods of preparation have now been studied by us:

(1) Direct precipitation, utilizing acetone, alcohol, benzoic acid, flavianic acid and tannic acid, both alone and combined.

(2) Fractional precipitation, utilizing $(NH_4)_2SO_4$, KI plus NaCl, precipitation at the isoelectric point, precipitation by CO_2, precipitation by dialysis and combinations of these methods.

(3) Adsorption and elution, utilizing kaolin, Lloyd's adsorbent, aluminium hydroxides A and B prepared as described by Willstätter, "Fasertonerde", franconite and benzoic acid.

(4) Coagulation and elution, utilizing acetone, alcohol and heat.

(1) *Direct precipitation*

(a) *Precipitation by acetone.* The method of precipitation by acetone was described by Zondek & Sulman [1937]; 1 mg. of the acetone-powder contains 1–3 P.A.U.[1]), the concentration depending on the titre of the original serum. The loss by precipitation amounts to 10 % at the most. This method of preparation renders storage of antigonadotropic factor in the desiccator without loss in titre for more than a year possible. Rabbit antisera treated by this method yield an acetone-powder which dissolves readily in water and normal saline giving concentrations of active substance 3–4 times greater than in blood. The acetone-powder from goat antisera, on the other hand, is difficultly soluble even to the original blood concentration, and its solutions always retain a slight opalescence.

(b) *Precipitation by alcohol.* The technique of the alcohol precipitation is the same as that of the acetone method. The results with alcohol are not as good,

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[1] 1 P.A.U. = 1 prolan anti-unit is the smallest amount of antiprolan able to neutralize the gonadotropic effect of 1 R.U. of prolan. At least 10 units must be assayed in a test rat [cf. Zondek & Sulman, 1937].

however, since the alcohol-powder is only incompletely soluble and the solutions are more opalescent than are those obtained with the acetone-powder.

(c) *Precipitation by benzoic acid.* On addition of a concentrated solution of benzoic acid in acetone to antiserum a precipitate is formed. This precipitate can be freed from benzoic acid by rinsing in acetone and the residue dried with ether. The dry powder thus obtained is difficultly soluble in water or normal saline, and is no richer in antiprolan than the material obtained in the simpler direct precipitation with acetone.

(d) *Precipitation by flavianic acid.* With a few drops of 10% aqueous flavianic acid maximum precipitation of a 1 : 10 dilution of prolan antiserum is obtained. After 48 hr. in the ice chest, such a precipitate was centrifuged and freed from flavianic acid by washing with 0·5% ammonia in 96% alcohol. The residue was dried by washing first with acetone and then with ether. The dry powder obtained was partly soluble in distilled water. The water-soluble portion (A) was rich in protein but poor in antiprolan, and could be refractionated by salting out with 50% sat. $(NH_4)_2SO_4$ into two components both of which contained but little antiprolan. The water-insoluble portion (B) dissolved in Locke solution (as well as in $N/50$ ammonia and buffers of pH 9·5) giving a solution which was poor in protein, could not be further fractionated with $(NH_4)_2SO_4$ and was no richer than A in antiprolan. Quantitative analysis showed that the flavianic acid precipitated only 50% of the total antiprolan and that this percentage was distributed equally between fractions A and B of the precipitate. The method described is not therefore promising.

(e) *Precipitation with tannic acid.* The addition of a few drops of a 10% aqueous tannic acid to prolan antiserum brought about maximal precipitation. The sedimented material proved to be rich in protein as well as in antiprolan, but attempts to separate the two were unsuccessful.

Of the five methods of antiprolan precipitation tested, therefore, the acetone method alone gives satisfactory results.

(2) *Preparation by fractional precipitation*

(a) *Fractional precipitation with sat. $(NH_4)_2SO_4$.* The results obtained with this method were described by Zondek & Sulman [1937]. Rabbit serum fibro-globulin (25% sat. $(NH_4)_2SO_4$) and euglobulin (32% sat. $(NH_4)_2SO_4$) fractions were found to be free of antiprolan, the whole of which was contained in the pseudoglobulin (48% sat. $(NH_4)_2SO_4$) fraction; transition fractions (32–48% sat. $(NH_4)_2SO_4$) contained antiprolan only in proportion to their contents of pseudoglobulin.

The same experiments when carried out with goat antiserum gave different results in that with this serum about 50% of the antiprolan was brought down with the euglobulin, and only 50% with the pseudoglobulin fractions [cf. Harington & Rowlands, 1937]; as in the rabbit the fibroglobulin and albumin fractions contained no antiprolan. Our results with salting out in diluted antisera were not promising, but with acetone-powder from rabbit antisera salting out was successful in antiprolan solutions of twice the physiological concentration: at 9·1% sat. $(NH_4)_2SO_4$ a very light precipitate containing no antiprolan separated; at 18·2% no further precipitate was obtained but at 23·1% saturation a heavy inactive precipitate formed; at 28·6% saturation a further light precipitate containing the entire antiprolan content of the antiserum was obtained; further inactive precipitates were obtained at 33·3% saturation. The fractional

$(NH_4)_2SO_4$ precipitation method in acetone-powder solution is effective therefore for concentrating the antigonadotropic factor in rabbit antiserum. Experiments with goat antisera indicated that this method may not give the same favourable results with antisera from other species.

(b) *Fractional precipitation with KI plus NaCl.* In 1 ml. prolan antiserum diluted with 2 ml. distilled water was dissolved 1 g. KI; the resulting solution was treated with 1 g. NaCl which did not entirely dissolve. On standing in the ice chest for 24 hr., a light precipitate containing about 50 % of the serum antiprolan separated out. The supernatant, after dialysis through a cuprophane membrane, was found to have retained 50 % of the total serum antiprolan. The antiprolan yield obtained in this method of precipitation is therefore rather low.

(c) *Fractionation by isoelectric precipitation.* The precipitate formed by native rabbit or goat prolan antiserum at the isoelectric point contains practically no antiprolan. By working with solutions poor in electrolytes (solution of acetone-powder in distilled water) and at high dilutions (1 : 25 or 50) and by acidifying gradually with acetic acid, however, a considerable concentration of antigonadotropic factor was possible. Success in the formation of a maximal precipitate at the isoelectric point is chiefly a matter of practice. With experience it is possible to follow the general progress of the precipitation by rough nephelometric estimation. Addition of bromocresol green facilitates both the nephelometric comparison and the determination of the optimum pH (approx. 5·5) for precipitation.

Table I shows that the antiprolan content of the isoelectric precipitate depends on the degree of dilution of the solution of acetone-powder.

Table I. *Recovery of antiprolan by isoelectric precipitation of solutions of acetone-powder from rabbit antiserum solution containing 50 mg. powder = 100 P.A.U. per ml.*

Dilution	Yield of antiprolan in isoelectric precipitate %	Dilution	Yield of antiprolan in isoelectric precipitate %
1 : 1	0	1 : 20	83
1 : 2	33	1 : 25	90
1 : 4	50	1 : 30	90
1 : 8	66	1 : 40	90
1 : 16	75	1 : 50	90

It follows from the table that for a maximal yield of antiprolan in the isoelectric precipitation of rabbit antisera, preliminary dilution of the acetone-powder solution to at least 1 : 25 is desirable. Dilution beyond 1 : 50 is not to be recommended owing to the difficulty of determining the point of optimal precipitation. The isoelectric precipitates obtained are not readily soluble and it may be preferable to take them up in Locke solution. With goat antisera acetone-powder the isoelectric precipitation method has failed as yet to give as favourable results as are given by the rabbit antisera; even at dilutions of 1 : 50, only 50 % of the total antiprolan content came down in the isoelectric precipitate. Isoelectric precipitation is therefore an effective method of concentrating antigonadotropic factor, but is not applicable to all species of antiprolan sera. It is specially suited, however, for the purification of rabbit antisera, a concentration to 6 P.A.U. per mg. of dry substance being obtainable.

(d) *Fractional precipitation by treatment with CO_2.* On passing CO_2 through antiprolan solutions, precipitation of part of the serum globulins occurs. The centrifuged precipitate contains at best about 40% of the total serum antiprolan. Concentration of antiprolan by this method is therefore impracticable.

(e) *Fractional precipitation by dialysis*. Native rabbit antiserum was dialysed in a cuprophane membrane against running tap water for 24 hr. The removal of electrolytes caused partial precipitation of the globulins and with them of 50 % of the serum antiprolan. With goat antisera also rapid precipitation of serum globulins containing 50 % of the total antiprolan was obtained. Fractional precipitation by dialysis is therefore impracticable as a method of concentrating the antigonadotropic factor.

(f) *Combined methods of fractional precipitation*. Two combined methods were tested: method A in which the isoelectric precipitation of rabbit antisera as described under (2 (c)) is followed by dialysis of the precipitate dissolved in Locke solution as described under (2 (e)) and method B in which the isoelectric precipitate (2 (c)) is extracted with 25 % sat. $(NH_4)_2SO_4$ (2 (a)) instead of with Locke solution.

(i) *Combined method A*. 50 mg. of antiprolan acetone-powder, corresponding to 1 ml. native rabbit serum (= 50 P.A.U.), were dissolved in 25 ml. distilled water and brought to maximal turbidity by addition of 1 % acetic acid drop by drop in the presence of bromocresol green. After 1 hr. the suspension was centrifuged and the precipitate was taken up in 3 ml. of Locke solution. This was then dialysed in a cuprophane membrane against running tap water for 24 hr. Under this treatment, a hydrophobic fraction of the isoelectric precipitate separated. The isoelectric precipitate without the hydrophobic fraction was found to contain 83 % of the total serum antiprolan. 1 mg. of this final material contained 10 P.A.U., the concentration attained being a tenfold one. This purified material when dissolved in 0·005 N NaOH and diluted to a concentration of 25 P.A.U. per 4 ml. gave slight ninhydrin and biuret reactions and but a slight clouding with acetone and salicylsulphonic acid; no coagulation occurred on boiling.

(ii) *Combined method B*. 50 mg. antiprolan acetone-powder, corresponding to 1 ml. native rabbit antiserum (= 50 P.A.U.), were dissolved in 25 ml. distilled water and brought to maximal turbidity by addition of 1 % acetic acid drop by drop in the presence of bromocresol green. After 1 hr. the precipitate was centrifuged, suspended in 3 ml. 25 % sat. $(NH_4)_2SO_4$ and shaken for 1 hr. The extract was dialysed against running tap water. A slight inactive precipitate separated. The remaining supernatant liquid contained 75 % of the total serum antiprolan at a concentration of 10 P.A.U. per mg. of dry substance, and behaved in protein tests as did the solution obtained in combined method A.

By combined fractional precipitation methods therefore, i.e. (a) isoelectric precipitation followed by dialysis, or (b) isoelectric precipitation followed by fractionation with sat. $(NH_4)_2SO_4$, a tenfold concentration of the antiprolan content of rabbit antiserum is possible.

(3) *Adsorption and elution*

(a) *Adsorption with kaolin and Lloyd's adsorbent*. The adsorbent was added to prolan antiserum at various pH values (4·5; 7·0; 9·5) until the reaction of the supernatant fluid to salicylsulphonic acid became negative. The suspensions were centrifuged and filtered and elution by means of glutamic acid, glycine and phosphate buffers at different pH values (9·5; 7·0; 4·9) was carried out. Elution of antiprolan from kaolin proved feasible only at alkaline or neutral reaction. Protein and antiprolan in the eluates gave parallel values. Elution from Lloyd's adsorbent failed consistently. These adsorption methods are therefore impracticable as methods of preparation of concentrated antigonadotropic factor.

(b) *Adsorption with Willstätter Al(OH)₃ A and B.* Antiprolan was adsorbed by alumina in alkaline, neutral and acid solution. Elution with alkaline, acid and neutral buffer solutions was tested. The acid eluents practically dissolved the aluminium compound giving toxic eluates which were useless for the purpose in hand; neutral buffers failed to elute antiprolan whilst alkaline buffers eluted varying amounts but at best not more than 30 % of the serum antiprolan. The methods described in this section are therefore of little practical value.

(c) *Adsorption with franconite and "Fasertonerde".* Adsorption by these two adsorbents occurred at every pH tested. Elution was possible only with alkaline buffers. The protein and antiprolan contents of the eluates gave parallel values, however, and it must be concluded that the method described is impracticable for the preparation of antiprolan in concentrated form.

(d) *Adsorption with benzoic acid.* Antiprolan solution was shaken with benzoic acid for 3 hr. The precipitate obtained was centrifuged, freed from benzoic acid by washing three times with acetone and tested for antiprolan. It was found to be inactive.

(4) *Preparation by coagulation and elution*

(a) *Coagulation with acetone and elution with dilute acetone.* As Zondek & Sulman have shown [1937], 80 % acetone precipitates the antigonadotropic factor. If water is then added to the suspension so that an acetone concentration between 40 and 45 % is obtained the antiprolan is eluted in quantities ranging between 33 and 50 % of the total available antiprolan. Simultaneously at least 33–50 % protein is redissolved. This method is therefore of no value for the preparation of antiprolan concentrates.

(b) *Coagulation with alcohol and elution with dilute alcohol.* Use of alcohol as described under 4 (a) for acetone gave similar results.

(c) *Coagulation by heat and elution with water.* The method here utilized has been used and is recommended by Besredka [1929] for the preparation of antisera concentrates. The antiprolan acetone-powder from rabbit serum was taken up in water in a concentration of 5 : 1. The mash obtained was shaken well and placed in the oven at 65° for 8 hr. to effect coagulation. The coagulate was extracted with distilled water. Since considerable quantities of protein accompanied the eluted antiprolan, such heat-coagulation followed by elution with water is not feasible as a method of separation of the antigonadotropic factor from serum protein.

DISCUSSION

The results presented in this paper show that the antigonadotropic factor cannot be obtained free from globulins by the common methods of adsorption and elution. This property distinguishes the antigonadotropic factor from numerous hormones and in particular from the gonadotropic factor itself. Evans *et al.* [1933], it will be recalled, adsorbed the gonadotropic factor on Willstätter alumina A and B and obtained highly purified hormone preparations on subsequent elution.

The antigonadotropic factor is always contained in the serum globulin, a property which places this factor in the same class as the antibodies. These experiments constitute further support for the view advanced that antiprolan is similar to an antibody and is not a hormone.

SUMMARY

Different methods of concentrating antiprolan are described.

I. Methods which appear to be of little practical value are:

(1) Direct precipitation with alcohol, benzoic acid, flavianic acid or tannic acid.

(2) Fractional precipitation utilizing potassium iodide plus sodium chloride, carbon dioxide or dialysis.

(3) Adsorption and elution, utilizing kaolin, Lloyd's adsorbent, Willstätter alumina A and B, franconite, "Fasertonerde" or benzoic acid.

(4) Coagulation and elution utilizing acetone, alcohol or heat.

II. The methods which were found to give results of significant practical value are:

(5) Precipitation and preservation by treatment with acetone and ether.

(6) Fractional precipitation with saturated ammonium sulphate.

(7) Isoelectric precipitation from dilute solutions poor in electrolytes.

The purest preparations were obtained by combined methods of preparation, viz.:

(8) Combined method A. Isoelectric precipitation of diluted antiserum, followed by solution of the precipitate in Locke solution and dialysis.

Combined method B. Isoelectric precipitation of diluted antiserum, followed by partial solution of the precipitate in 25 % sat. $(NH_4)_2SO_4$ and dialysis of the filtrate.

The concentrated antiprolan solutions obtained by the combined methods of preparation gave only a faintly positive reaction in the ninhydrin and biuret tests, developed only slight turbidities with acetone and salicylsulphonic acid and did not coagulate on boiling; 1 mg. purified antiprolan dry substance contained 10 antiprolan units.

The combined methods of preparation were not of equal efficacy with the two species tested, the results obtained with rabbit antiserum by the combined methods being far more promising than those obtained with goat antiserum.

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CCXLIV. ESTIMATION OF THE ANTI-HAEMORRHAGIC VITAMIN

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IN a former paper [Almquist & Stokstad, 1937] it was stated that chicks fed on diets containing low levels of vitamin K tended to show a maximum blood clotting time at 2 weeks of age and a decreasing blood clotting time after this age. The method of preventive assay and the use of a reference standard, as well as a negative control, were briefly illustrated. Further studies of this assay procedure have been made and may now be reported.

METHODS AND RESULTS

The feeding and housing of the chicks and the determination of blood clotting times were identical with the procedures already described [Almquist & Stokstad, 1937]. In withdrawing blood, incisions about 1 mm. long were made with a fine pointed scalpel in a wing vein which was exposed near the junction of the ulna, radius and humerus.

The sole source of supplementary vitamin K used in these experiments was a hexane extract of alfalfa which has been adopted as a reference standard in our vitamin K assays. 1 ml. represented 1 g. dried alfalfa.

Chicks from hens on diets containing approximately the same vitamin K content were divided into groups and given the basal diet plus various levels of the standard vitamin K sources. The results of one such experiment are given in Table I. The marked tendency toward a maximum blood clotting time at 2 weeks of age is a most striking feature of these results.

Table I. *Average blood clotting times in relation to dietary vitamin K level and age of chicks. Exp. I*

Standard vitamin K soln. per kg. diet (ml.)	No. of chicks	Average blood clotting times in min. at age of			
		1 week	2 weeks	3 weeks	4 weeks
2·0	10	20·2 ±5·6	Greatly prolonged	10·1 ±1·2	8·9 ±0·6
3·0	10	10·3 ±2·3	19·5 ±3·0	7·1 ±0·8	7·0 ±1·0
4·0	10	6·8 ±1·0	7·6 ±0·7	5·8 ±0·9	4·8 ±0·6
5·0	10	5·7 ±0·6	6·3 ±0·9	5·2 ±0·6	4·2 ±0·4
10·0	10	—	Greatly prolonged*	4·2 ±0·8	4·0 ±0·5
50·0	10	3·4 ±0·4	3·5 ±0·4	3·4 ±0·4	2·7 ±0·3

* This group had received only the basal diet for 2 weeks; all individuals were markedly deficient. They received the vitamin K supplement at an age of 2 to 4 weeks.

In a second experiment of this nature we obtained chicks from hens receiving diets rich in vitamin K. Day-old chicks from this source had an average blood clotting time of 1·9 min. as compared with 3·2 min. for chicks from the sources used in the first experiment. These chicks with a high reserve of vitamin K did

not show such a marked increase in blood clotting time when they received less vitamin K in the diet as those of the first experiment, although there was a definite increase during the second week. Apparently, the high vitamin K reserve of these chicks was sufficient to compensate for the low vitamin K intake through the critical period up to 2 weeks of age. The results are given in Table II.

Table II. *Average blood clotting times in relation to vitamin K level and age of chicks. Exp. II*

Standard vitamin K soln. per kg. diet (ml.)	No. of chicks	Average blood clotting time in min. at age of			
		1 week	2 weeks	3 weeks	4 weeks
2·0	16	5·2 ±0·8	9·6 ±1·9	10·9 ±1·7	8·0 ±1·1
2·5	16	3·5 ±0·6	8·3 ±1·2	9·4 ±1·2	—
3·0	15	3·4 ±0·2	7·2 ±1·1	7·8 ±1·4	—
4·0	15	2·4 ±0·2	6·6 ±1·2	6·9 ±0·9	6·5 ±0·3
5·0	15	2·6 ±0·2	5·1 ±0·5	4·0 ±0·3	—
10·0	15	2·7 ±0·3	3·0 ±0·3	3·0 ±0·2	—
50·0	15	2·4 ±0·3	3·9 ±0·3	3·2 ±0·2	—
0·0	6	—	Greatly prolonged	—	—

In a third experiment chicks from the same source as in Exp. I were used with a repetition of the results of Exp. I. There was again the well-defined tendency toward a maximum blood clotting time at 2 weeks of age, with many individuals of the lowest vitamin K intake having clotting times that were greatly prolonged. About 15 chicks per group were used in the last two experiments.

The blood clotting times of chicks at 3 weeks of age in these experiments are given in Table III. It is apparent that, although certain groups of chicks in

Table III. *Average blood clotting times at 3 weeks of age of chicks in Exps. I, II and III*

Standard vitamin K soln. per kg. diet (ml.)	Average blood clotting time in min.		
	Exp. I	Exp. II	Exp. III
2·0	10·1 ±1·2	10·9 ±1·7	12·5 ±1·4
2·5	—	9·4 ±1·2	8·6 ±0·8
3·0	7·1 ±0·8	7·8 ±1·4	7·0 ±0·8
4·0	5·8 ±0·9	6·9 ±0·9	5·7 ±0·5
5·0	5·2 ±0·6	4·0 ±0·3	4·8 ±0·6
10·0	4·2 ±0·8	3·0 ±0·2	4·1 ±0·2
50·0	3·4 ±0·4	3·2 ±0·2	—

Exps. I and III had passed through a period of markedly prolonged blood clotting time and those of Exp. II had not done so, all groups at 3 weeks of age had approximately the same blood clotting time for the same vitamin K level in the diet. It appears that at this age the chicks had achieved a balance with respect to the vitamin K content of the diet.

During Exp. III, three groups of chicks, all with greatly prolonged individual clotting times, were given at 2 weeks of age diets containing 3 ml., 5 ml. and 10 ml. of the standard solution per kg. The average clotting times for these groups at 3 weeks of age were, respectively, 6·9, 5·2 and 3·8 min., while the average clotting times of contemporary groups which had received these vitamin K levels continuously from 1 day of age, were, respectively, 7·0, 4·8 and 4·1 min. These results show that, in spite of a previous condition of severe depletion, the chicks were able, within 1 week or less, to attain an average blood clotting time which depended principally upon the vitamin K level in the diet.

In order to make certain that the actual consumptions of vitamin K were comparable from group to group, careful records of food consumption and growth were kept. These data may be effectively summarized by the statement that growth and food consumption were not appreciably different within the groups of any one experiment and, in fact, did not vary to any significant extent in the separate experiments. This, of course, is consistent with the fact that vitamin K is not a growth factor.

It was stated in a former paper [Almquist & Stokstad, 1937] that there was no evident relation between vitamin K deficiency and haemoglobin level in non-haemorrhagic individuals. Exception to this statement has been taken by Thayer *et al.* [1937] who have reported restoration of both blood clotting power and haemoglobin level after the administration of vitamin K to deficient chicks, but who did not distinguish in their report between haemorrhagic and non-haemorrhagic individuals. It is well known that haemorrhagic chicks suffer from anaemia induced by loss of blood into the tissues and that within 2 or 3 days after administration of adequate doses of vitamin K the blood may be reabsorbed and the haemoglobin level restored toward normal.

During the course of these and other studies, haemoglobin measurements were made on the blood of individual chicks with prolonged clotting time. Measurements were made in these cases with the New Dare Haemoglobinometer, the mean of 5 or more closely agreeing readings being taken as the final value. Eleven chicks, 2 weeks old, with individual clotting times over 60 min. in each case, were found to have haemoglobin levels ranging from 8·10 to 9·60 g. per 100 ml. with a mean of 8·56. None of these chicks were afflicted with visible haemorrhages. The mean value compares favourably with one of 7·97 obtained by Harmon [1936] with the identical instrument in the case of 2-week old normal chicks fed on a practical ration. Four additional non-haemorrhagic chicks with individual clotting times greater than 60 min. were tested at an age of 3 weeks. The haemoglobin levels found were 7·8, 8·4, 9·0 and 9·7 g. per 100 ml. These values are far above what may be considered an indication of anaemia.

One of the most serious obstacles to accuracy in vitamin K assay by any procedure is the large variability of blood clotting time encountered, although source of chicks, level of vitamin K intake and reserve of the chicks are made as nearly uniform as possible. This variability is not appreciably lowered by previous severe depletion of the chick. It remains high even in chicks that have all received the same amount of the vitamin in the diet for as long as 5 weeks and that have grown equally well.

In a search for other factors influencing the blood clotting time, we measured the clotting times of chicks maintained on the same low dietary vitamin K level, in certain individuals only after complete anaesthesia induced by subcutaneous injection of sodium pentabarbital (Nembutal), and in others in the usual manner. A reduction in blood clotting time due to a condition of fright seemed possible. Such an effect would probably be minimized in birds that had been under the influence of a powerful anaesthetic for ½ hr. or more. However, over a range of 6–19 min., no appreciable differences in average clotting time were found.

Second samples of blood taken immediately after the first samples and from the same incision usually clotted in slightly less time than the first samples. This was also found to be the case for first and second samples taken from different incisions, one on each wing of the chick. In no case was more than approximately 0·2 ml. taken per sample. Since there was no appreciable loss of blood while these samples were being taken, the quantity of blood lost could scarcely have exerted any influence on the blood clotting time. It does not

appear that this reduction in clotting time was due to contamination of the second blood sample by fluids from the cut tissues, since the same slight shortening of the clotting time was observed whether the samples were taken from one cut or from different cuts in the same bird. Furthermore, the incisions were made in such a manner as to avoid cutting of tissue other than the vein.

In a further study of this shortening of the clotting time it was found that the differences in the average clotting times of the first and second blood samples lacked significance in the statistical sense, although there was a consistently shorter average time for the second samples.

None of the above findings aid in explaining the large variability in blood clotting times in chicks of the same group. It is logical to expect that a large part of such variability may be caused by individual differences in the chicks in regard to their vitamin K metabolism. In an attempt to secure a measure of such individual source of variability, we have obtained at intervals of a few days the blood clotting times of 57 identified chicks maintained on the same diet low in vitamin K. Data illustrating the characteristically different blood clotting times of certain chicks reared together upon the same diet and vitamin K level are given in Table IV.

Table IV. *Blood clotting times in minutes of individual chicks fed on the same diet low in vitamin K*

	Age in days			
Chick no.	21	24	27	29
5370	>30	>30	>30	>30
5389	24·4	>30	>30	26·0
5392	>60	>30	21·5	>30
5360	7·0	7·1	4·7	5·4
5378	2·9	5·7	2·8	3·6
5386	13·4	9·7	8·6	10·5

This group of chicks was of random selection. Individuals in the group grew at rates which varied over a twofold range. A small positive correlation between chick weight and blood clotting time was indicated by the coefficient, 0.276 ± 0.086, which is barely significant. This tendency for blood clotting time to be influenced by rate of growth can be almost entirely obliterated by selecting chicks of uniform size at 1 week of age.

Since it has been shown by Greaves & Schmidt [1937] that lack of bile interferes with the absorption of vitamin K in the rat, it seemed possible that chicks in a state of chronic deficiency might be cured by addition of bile acids to their diet, if one cause of their deficient condition were poor absorption of vitamin K. Experiments were conducted using chicks of prolonged blood clotting times; however, the addition of the bile acids, cholic acid, deoxycholic acid, dehydrocholic acid and taurocholic acid to the diet at a level of 0·5 % had no detectable effect on the average blood clotting time after 1 week of such feeding, although a uniform, nearly adequate level of vitamin K was present in the diet. Control groups also showed no appreciable or general changes. The characteristic clotting time differences, as illustrated in Table IV, were evidently not due to a lack of bile acids in the digestive tract.

As a result of the large individual variability of chicks in regard to blood clotting time, it becomes evident that a reduction of the probable errors in average blood clotting time requires primarily an increase in the number of chicks per test group. This means that any procedure requiring complicated and lengthy

treatment of each animal and of each blood sample will be extremely laborious as well as more susceptible to experimental errors. For these reasons we have sought to perfect the most simple method of conducting a vitamin K assay.

In the procedure now adopted by us 10 chicks (more when greater accuracy is desired) that have been maintained on the basal diet for 1 week since hatching are placed in each test group and fed on a supplemented basal diet from 7 days to 14 or 21 days. The purposes served by maintaining the chicks for 1 week on the basal ration are many, e.g. depletion of the reserve, detection of weak, defective and slow-growing chicks and elimination from the test pens of most of the usual early chick mortality. Included with the test groups receiving the supplement intimately mixed with the diet are a negative control group having no supplementary vitamin K in the diet and several groups on diets containing various levels of the reference standard.

The reference standard could, of course, be assigned a certain "unit" value, but we have preferred not to follow the common tendency to define "units" and possibly lead vitamin K into the confusion of meanings and definitions that has been troublesome in the case of certain other vitamins. It would seem better to defer such definitions until requirements can be based upon a weight of a pure or standard preparation.

As previously shown, the age of 14 days is one of maximum susceptibility to inadequate vitamin K intake. An assay can therefore be terminated at this time, after 7 days of supplementary feeding, provided that a series of suitable reference standard control groups are available and all chicks are of a common origin and have received the same treatment.

For more precise assay it is preferable to continue the supplementary and control feeding to 3 weeks of age. As indicated in Table III, chicks at this age seem to attain a final adjustment of blood clotting time to the vitamin K level of the diet. The results of Table III have been used in constructing Fig. 1, which expresses the relation of the logarithm of the vitamin K concentration in the diet to the reciprocal of the average blood clotting time. The relation is evidently linear, which means that the clotting power of the blood, as expressed by the reciprocal of the clotting time, is a simple function of the logarithm of the vitamin K concentration in the diet. The line relating the reciprocal of the clotting time to the logarithm of the dietary vitamin K concentration appears to extrapolate toward the origin. This implies that, as the vitamin K level is decreased, blood clotting time tends to become extremely prolonged.

Fig. 1. Relation of the reciprocal blood clotting time to the logarithm of the vitamin K level in the diet (ml. reference standard sol. per kg. diet).

For the curve given it was found that

$$\text{log vitamin K concentration} = \frac{3 \cdot 6}{\text{blood clotting time}}.$$

It is probable that the constant in this equation will not hold in all cases for chicks of this age and that the interpolation of the results of any assay should be made separately for each series. Although the same equation was obtained from the results at 4 weeks, the constant was definitely lower, approximately 2·8.

This constant would be expected to decrease with increasing age of the chicks.

The linear relation of the logarithm of the dietary vitamin K level to the reciprocal of the blood clotting time affords a very useful means of interpreting average blood clotting times of test groups in terms of the potency of the reference standard. Thus, for interpolating the results of an assay it is necessary to have only two accurately determined points from reference standard groups.

It appears inadvisable at present to specify certain values for blood clotting times to serve as a basis for defining units or activity of vitamin K concentrates, as Thayer *et al.* [1938] have proposed. These workers have defined a unit of vitamin K as that quantity of material required to reduce the clotting time of the blood of 50 % of the chicks to 10 min. or less. A clotting time of 10 min. or less was considered normal. It is only necessary to recall the variability of blood clotting time with age (Tables 1 and II) to realize that highly erroneous results can be obtained by such method of assay unless the ages of chicks and the vitamin K reserves of chicks are closely standardized.

The fact that on low dietary levels of vitamin K chicks with only a moderate K reserve tend to show at 2 weeks a maximum blood clotting time, which later decreases, while chicks with a greater reserve may not show such marked changes, has several interesting implications. The day-old chick weighs 30–40 g. and has a corresponding, definite and increasing requirement for essential food factors. However, the intake of such factors from the diet starts at zero from which it increases as the chick consumes food. The chick must soon obtain entirely from the diet the essential factors which it may have carried in reserve at the time of hatching. During the first days of the chick's life, therefore, the reserve is being depleted pending the acquisition of a sufficient quantity of essential food factors from the diet. When one of these food factors is limited by a low intake in the diet, the possibility of deficiency is greatly increased and, when the reserve of the chick is also low, a seriously deficient condition is almost certain.

During the period from 1 to 2 weeks the chick makes the maximum gains per unit of food consumed. It is during this period of most rapid gain in relation to food consumed that the chick is most affected by an inadequate dietary supply of vitamin K. Even in Exp. II, where the chicks had a high reserve of vitamin K, it was found that the greatest increase in blood clotting time took place during the second week. It is significant, in this respect, to note that Harmon [1936] has shown that at an age of 2 weeks the haemoglobin levels of normal chicks pass through a minimum value which is elevated again at 3 weeks.

After an age of 2 weeks the gain in wt. per unit of food consumed decreases and it is found that the blood clotting time also drops, indicating that the requirement of vitamin K per unit of diet has decreased. This decrease is probably analogous to the lowering of the vitamin G dietary levels required by chicks [Heuser *et al.*, 1938].

SUMMARY

1. The blood clotting time of the chick varies with the age and the vitamin K reserve in the chick, and the vitamin K level in the ration, and tends to reach a maximum at 2 weeks of age.

2. At 3 weeks of age the chick achieves a balance of blood clotting power with respect to the vitamin K level in the ration.

3. The reciprocal of the blood clotting time is a simple linear function of the logarithm of the vitamin K level in the ration, over a practical range of values.

4. Chicks free from haemorrhage but with greatly prolonged blood clotting times had normal haemoglobin levels.

5. An improved assay procedure for vitamin K is suggested.

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CCXLV. THE TIME FACTOR IN THE INTERACTION OF AMINO-ACIDS WITH SUGARS

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In a previous communication we have shown [Frankel & Katchalsky, 1937] that the interaction of α-amino-acids and peptides with sugars in slightly alkaline, neutral and slightly acid solution leads to a decrease in pH owing to the disappearance of basic amino-groups, but no quantitative conclusions were drawn. It was mentioned that the interaction in question "proceeds over a relatively long time interval". In the experiments now reported the time progress of the decrease in pH is described in greater detail.

The study of the time factor throws light also on a recent paper by Balson & Lawson [1938] who, on the other hand, state that the pH decreases shown by amino-acids or peptides in mixture with sugars is solely attributable to the acidic properties of the sugars themselves. The discrepancy between the results of Balson & Lawson and our own is, we believe, due mainly to the fact that, in choosing their experimental technique, these authors failed to take into account the time factor of the interaction. The interaction between the aldoses and amino-acids or peptides becomes noticeable, under certain conditions, only after some time. In the experiments described by us intervals of 20–30 min. were allowed between NaOH additions in the potentiometric technique, and observations in the colorimetric experiments were extended over periods of 24–48 hr. Balson & Lawson, on the other hand, used their special technique [1935] which enables them to shorten even that time interval which is normally required in potentiometric titrations between consecutive additions, and thus created markedly unfavourable conditions for observations of the interaction.

Other minor causes of the discrepancy are dealt with later in this paper.

In order to study the time factor more fully we carried out the present experiments as follows.

I. Mixtures of amino-acid and sugars were titrated by the potentiometric method under different time conditions: in (1) the interval between each addition of NaOH was 10 min., in (2) the interval was 3 hr. Time in each case was counted from the moment at which initial potential was attained.

II. Equal parts of mixtures containing both components of the reaction were brought to desired pH values by addition of NaOH and both the initial pH and the changes of pH were determined electrometrically during a period of several hours.

In all cases controls containing either the amino-acid or the sugar at the pH and concentration of the test mixtures, were run.

The amino-acid was glycine. The sugars were: sucrose and fructose for non-aldehydic, non-reactive sugars; glucose and galactose for the aldehydic sugars. The experiments were carried out under sterile conditions at 20° throughout.

RESULTS

I. *Potentiometric titrations with different time intervals between NaOH additions*

(a) *Glycine + non-aldehydic sugars*

1. *Time interval between additions—10 min.*

When the slight correction necessitated by the acidity of the sugars is allowed for (0–3 mV. for sucrose, 0–6 mV. for fructose) the curves obtained are identical with that of glycine + water.

2. *Time interval—3 hr.*

Once the potentials are established no change in pH occurs. The titration curves of the solutions are therefore the same whether the NaOH is added at intervals of 10 min. or 3 hr.
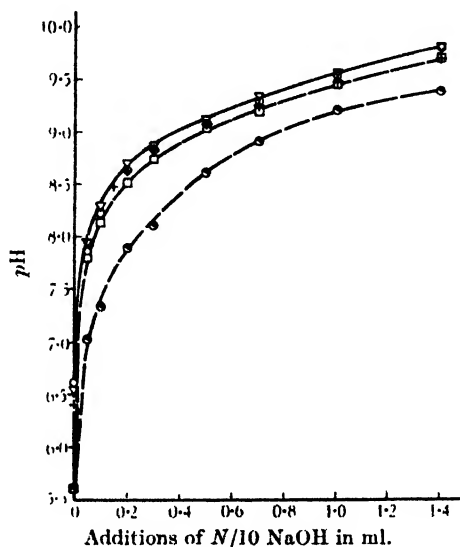
Fig. 1. Titration curves of glycine-sugar mixtures with different time intervals between NaOH additions. ▽ 1 ml. glycine $M/4 + 1$ ml. H_2O. ○ 1 ml. glycine $M/4 + 1$ ml. sucrose $M/1$ (time interval 10 min. or 3 hr.). + 1 ml. glycine $M/4 + 1$ ml. fructose $M/1$ (time interval 10 min. or 3 hr.). □ 1 ml. glycine $M/4 + 1$ ml. glucose $M/1$ (time interval 10 min.). ⊘ 1 ml. glycine $M/4 + 1$ ml. glucose $M/1$ (time interval 3 hr.).

Table I. *Potentiometric titration of 2 ml. of mixtures containing glycine* M/8 *and glucose* M/2 *by NaOH* N/10, *each addition of alkali being made (1) 10 min. or (2) 3 hr. after the initial potential was attained.*

Additions of alkali (ml.)	pH	
	(1)	(2)
0·0	5·61*	5·61*
0·05	7·71	7·03
0·1	8·13	7·34
0·2	8·51	7·89
0·3	8·73	8·11
0·5	9·04	8·61
0·7	9·21	8·91
1·0	9·47	9·21
1·4	9·72	9·41
1·8	9·96	9·90

* The measured pH; a decrease in pH might have occurred here during the mixing and measuring.

(b) Glycine + aldehydic sugars

1. *Time interval—10 min.*

In this case also the necessary correction for the acidity of the glucose is small (0–4 mV.) within the pH range and concentration ratio of the experiments.

Even when the potentiometric titration was conducted at time intervals of 10 min. the potentials initially attained tend to decrease. Yet the decrease under these conditions (Δ pH about 0·1), though greater than the range of error, is still rather small (Fig. 1; Table I (1)). Balson & Lawson [1938] correctly concluded that a pH lowering of this size is too small for an interaction to be inferred.

2. *Time interval—3 hr.*

The pH values found after each addition were lower than the corresponding values obtained with additions at intervals of 10 min. by as much as 0·8 pH. (Cf. Fig. 1, Table I (2).)

II. *Electrometric pH measurements during 3 hr. in mixture solutions brought by additions of NaOH to different initial pH values*

(a) Glycine + non-aldehydic sugars

The sugars were either sucrose or fructose.

All pH values remained constant at their initial level for 3 hr. Sterile mixture solutions at pH up to 8·8 and sterile acid and neutral controls underwent no change in pH within one or more days; both sugar controls and mixtures of higher alkalinity undergo pH depressions when kept for 24 hr. or more. This result is to be expected in view of the known instability of sugars in alkaline solution.

(b) Glycine + aldehydic sugars

Glycine + glucose. Marked and rapid decreases in the pH values were observed (Table II). The velocity of the depression decreased with increasing alkalinity. All pH values attained constancy at least 30 min. before the end of the observation period.

Table II. *Potentiometric measurements of pH changes with time in 2 ml. mixtures containing glycine M/8 + glucose M/2 + x ml. NaOH N/10*

Time after mixing min.	x = ml. NaOH N/10					
	0·05	0·1	0·3	0·5	1·0	1·8
			pH values			
25	7·71	8·13	8·73	9·04	9·43	9·95
30	7·63	8·08	8·71	9·02	9·40	9·93
37	7·47	8·01	8·68	9·01	9·40	9·89
45	7·34	7·94	8·65	8·99	9·40	9·89
60	7·23	7·89	8·64	8·99	9·40	9·89
75	7·17	7·78	8·61	8·95	9·38	9·89
150	7·17	7·47	8·47	8·88	9·36	9·87
180	7·17	7·47	8·46	8·88	9·35	9·87

In sterile mixtures and controls of pH < 8·3 additional decreases did not occur even after several days. In the alkaline range, above pH 8·3, glucose controls prepared and maintained under sterile conditions showed depressions in pH after one or more days showing decomposition of the sugar. The mixtures under the same conditions also showed additional depressions. It is obvious that

sugars which are allowed to stand for a longer period in strongly alkaline media undergo chemical changes which, as in certain cases observed by the authors and others, are aided by the presence of amino-acids. As has previously been shown, more strongly alkaline media induce a new type of reaction in the mixtures. It seems, therefore, advisable to reduce the observation time, which in the colorimetric experiments was extended over one or two days, to an interval of e.g. 3 hr. within which no decomposition of the sugars occurs.

It may be noted here that the observations on the diminution of the pH decrease at higher alkalinities were taken at points far enough from the end-point of the titration to be uninfluenced by the final convergence of the titration curves.

Galactose when mixed with glycine behaves in the same way as glucose, except that within a pH range of 7–8 the readings with galactose became constant within 20 min., whereas in the pH range 8–9 constancy is attained after 2 hr.

The molar concentration ratio of amino-acid to sugar in our previous experiments was 1 : 1, and in the mixtures the concentration of each component was $M/4$. The corrections for the acidity of the sugars in the pH range investigated were found to be between 1–2 mV. and were therefore practically negligible.

It may also be remembered that in our previous colorimetric experiments the acidity of the sugars was eliminated by equalizing the pH of both component solutions before mixing (principle of isohydric solutions).

Lastly it may be mentioned, in anticipation of a later paper, that leucyl-glycine, the peptide investigated by Balson & Lawson, is less reactive when mixed with aldoses than glycine or its peptides in which the free NH_2-group belongs to the glycine moiety.

SUMMARY

1. Mixtures of glycine and either non-aldehydic or aldehydic sugars were potentiometrically titrated under different time conditions.

No pH depression occurred in mixtures containing non-aldehydic sugars and no influence of the length of the intervals between NaOH additions on the titration curve was detectable. In mixtures containing glucose a pH depression appeared which was much more marked when the time interval between the NaOH additions was extended.

2. No change within at least 3 hr. in the pH values occurred in mixtures of glycine and non-aldehydic sugars brought to different initial pH. Under the same conditions marked and rapid decreases in the pH values occurred in mixtures containing glycine and either glucose or galactose.

3. The view of Balson & Lawson, that the pH depressions observed by us are to be attributed solely to the acidic properties of the sugars is shown to be incorrect.

4. Owing to different experimental conditions no direct conclusion in regard to our previous findings can be drawn from the results of Balson & Lawson.

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CCXLVI. THE EFFECTS OF ANAESTHETICS AND OF CONVULSANTS ON THE LACTIC ACID CONTENT OF THE BRAIN

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THAT disturbances in the intermediary carbohydrate metabolism of the brain may be involved in certain "functional" disorders of the central nervous system is indirectly suggested by many observations. The beneficial effect of the "insulin shock" therapy introduced by Sakel [1937] for the treatment of schizophrenia is a particularly striking example, since one of the effects of hypoglycaemia is to diminish the supply to the brain of glucose, the normal substrate for oxidative catabolism in this organ [Dameshek & Myerson, 1935].

In epilepsy, also, the brain metabolism may be abnormal. Some form of anoxaemia has long been considered a possible etiological factor in convulsive disorders [Cobb, 1936]. The seeming antagonism between epilepsy and schizophrenia led Meduna [1937] to introduce the metrazol convulsion therapy for schizophrenia.

An older theory of narcosis, recently given a new aspect by the work of Quastel and his coworkers [Quastel & Wheatley, 1932; 1934; Jowett, 1938], suggests that an interference in brain oxidations may be involved in narcosis. Such an interference may be important in the prolonged narcosis therapy for schizophrenia [Palmer, 1937]. The work of Quastel & Wheatley [1933] also suggests a toxic effect of amines on brain metabolism as of possible importance in mental disorder.

The observations of Loevenhart *et al.* [1929] on the temporary effects of cyanide injection and of inhalation of high carbon dioxide-air mixtures on schizophrenic patients are of interest also.

The mechanism of glucose catabolism in the brain is a very controversial subject at present. Whether lactic acid is a direct intermediate, or whether it arises from pyruvic acid as a side reaction, is not known with certainty. But in either case, a disturbance at any point in the reaction chain or chains involved in glucose catabolism might be expected to cause an increase or decrease in the concentration of lactic acid in the brain. The object of this investigation was to discover whether or not such an increase or decrease is caused by anaesthetics or by convulsant drugs.

With the exception of the work of Kerr and coworkers [1936; 1937; Avery *et al.* 1935], the brain lactic acid values given in the literature are not dependable, because of post-mortem glycolysis. Kerr and coworkers froze the brain *in situ* in the living animal, but their technique required the use of anaesthetics, which decrease the brain lactic acid, as will be shown.

EXPERIMENTAL

Mice were used in this investigation. These animals are small enough to be plunged into liquid air and frozen, without the use of anaesthetics or surgical procedures. The mouse is frozen solid in 2–3 sec., and reaches the temperature

of the liquid air in 60–90 sec. The brain is then removed, using chilled instruments and working on a surface consisting of a strip of cardboard chilled in liquid air. The tissue, instruments, and cardboard are chilled at intervals during the operation, so that the brain does not soften at any time. The whole brain is removed, and stored in liquid air, ready for crushing.

The crusher (Fig. 1) is similar to that described by Graeser *et al.* [1934], but much smaller, and simplified. The crushing surfaces are of tool steel; the ring and base of iron. The crusher is chilled by submerging for a few sec. in liquid air, the frozen tissue then placed on the anvil, with the ring in place, and the tissue crushed by several blows of a hammer on the steel plunger. The crushed tissue forms a solid disk which sticks in the iron ring, and is poked out with a glass rod into the $ZnSO_4$-H_2SO_4 contained in a tared centrifuge tube (with rubber stopper). In order to avoid loss of tissue during the transfer, a glass funnel with the stem cut off is used. The tube is restoppered, shaken well, and weighed.
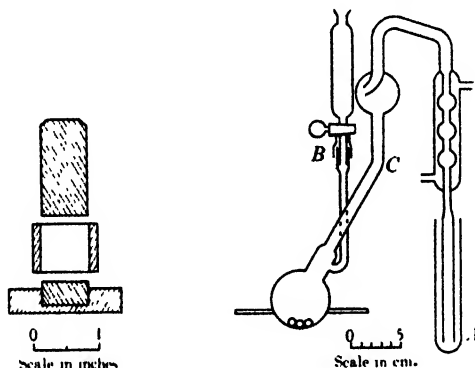
Fig. 1. Tissue crusher.   Fig. 2. Distillation apparatus.

Determination of lactic acid

Lactic acid was determined by a modification of the method of Friedemann & Graeser [1933]. Since a mouse brain weighs only 0·3–0·4 g., it was necessary to use much smaller quantities than those employed by Friedemann & Graeser.

The distillation was carried out in the pyrex glass apparatus shown in Fig. 2. All rubber connexions have been eliminated except that at B, which does not become heated, and here the rubber does not come into contact with the solution. The apparatus is easily rinsed by inverting and running water in at A and out at B. Draining through B is satisfactory unless the angle at C has been made too acute; 150° is about right. The apparatus is easily constructed from a 100 ml. distilling flask and the micro-Kjeldahl distillation apparatus described by Cavett [1931].

Protein removal is accomplished by the $Zn(OH)_2$ method. The crushed tissue is added to 3 ml. solution containing per litre 35·5 g. $ZnSO_4$, $7H_2O$ and 355·5 ml. N H_2SO_4 [Blatherwick *et al.*, 1935]. An amount of 0·5 N NaOH is added sufficient exactly to neutralize the acid solution (determined by previous titration, using phenolphthalein). Water is then added to make a total volume of 7·5 ml., counting the tissue 80 % water. The mixture is allowed to stand for 30 min. with occasional shaking, and centrifuged.

To 5 ml. supernatant fluid are added 0·6 ml. $CuSO_4$ (containing 200 g. $CuSO_4$, $5H_2O$ per l.), and 0·6 ml. $Ca(OH)_2$ suspension (prepared by slaking 200 g.

fresh lime and diluting to 1 l.). Water is added to the mixture to make a total volume of 15 ml. This is allowed to stand for 30 min. with occasional shaking, centrifuged and decanted.

A 13 ml. aliquot of the solution is introduced into the distillation apparatus (at B), followed by 2 ml. of H_3PO_4-$MnSO_4$ mixture (100 g. $MnSO_4$, $4H_2O$ and 25 ml. 85 % H_3PO_4 per l.), and 5 ml. water. The funnel is then connected at B, with the stopcock closed, and filled to the 15 ml. mark with a colloidal suspension of MnO_2 (freshly prepared by mixing equal quantities of 0·013 % $KMnO_4$ and 0·03 % $MnSO_4$, $4H_2O$ solutions. Rapid flocculation does not occur unless $MnSO_4$ is slightly in excess). The receiver contains 2 ml. 0·1 M $NaHSO_3$.

The solution is brought to boiling, and small portions of MnO_2 are run in at intervals, the whole 15 ml. being added during a 10 min. period. The total distillation period is 15 min., the rate being such as to give a final vol. of 20 ml. in the receiver. At the last, the receiver is lowered and the distillation continued for about 1 min. to rinse the outlet. Bumping is prevented by the presence of three glass beads in the flask. After the distillation, the receiver is cooled in ice water.

The excess $NaHSO_3$ is removed by 0·3 N I_2, and the end-point adjusted with 0·005 N $Na_2S_2O_3$ and 0·0025 N I_2, using 1 ml. 1 % starch indicator. The bound $NaHSO_3$ is then released by adding 5 ml. saturated Na_2HPO_4, and titrated with standard 0·0025 N I_2 (containing 10 g. KI per l.). A 2 ml. micro-burette is used, and an air-driven stirrer facilitates the titration.

1 ml. 0·0025 N I_2 is equiv. 0·1125 mg. lactic acid.

Purification of the reagents used is necessary. Blank determinations usually required about 0·1 ml. of 0·0025 N I_2.

Tests on known lactic acid solutions (prepared from Zn lactate) in amounts covering the range involved in these experiments indicated that the results were accurate to within ± 3 mg. per 100 g. of brain tissue. Since this is much smaller than the differences between individual normal mice, the method is sufficiently accurate for the present purpose.

Blank tests on phenobarbital, ether, picrotoxin, metrazol, nicotine and NaCN in amounts likely to be present in experiments reported here, showed that these drugs do not form appreciable amounts of $NaHSO_3$-binding substances.

Brain lactic acid in normal mice

Table I gives the data obtained on normal mice. Brain lactic acid values in mice not exercised before killing ranged from 12 to 25 mg. per 100 g. brain tissue (wet wt.), with a mean of 18·9 mg. per 100 g. Neither body wt. nor sex influenced the results significantly. Most of the mice were not subjected to a period of food deprivation before the experiment; such a period was found to have no influence on the brain lactic acid.

It was found that a period of strenuous exercise increased the brain lactic acid significantly. The mouse was placed in a covered beaker, which was then continuously tilted, shaken and rotated, forcing the mouse to exercise in attempting to right itself and maintain equilibrium. The brain lactic acid in these mice was 25–28 mg. per 100 g., with a mean of 26·8, an average increase of 7·9 mg. per 100 g. due to the exercise.

Since in strenuous exercise lactic acid accumulates in the blood, it was desirable to determine whether the increased brain lactic acid was due to diffusion from the blood into the brain. Three mice were injected with 4–8 mg. lactic acid (as Na salt) in 0·56 M solution, by tail vein, and killed 5 min. later. The brain lactic acid values agreed with those of normal resting mice; hence it is

concluded that the extra brain lactic acid arising during exercise has its origin in the brain itself, and is due to increased activity of this organ. These observations are in harmony with those of Dameshek & Myerson [1935], who found no absorption by the brain of lactate from the blood in patients in insulin hypoglycaemia (during which brain lactic acid is low). Contrary observations have been made by others, however [McGinty, 1929; Himwich & Nahum, 1932].

Two experiments showed that adrenaline does not increase the brain lactic acid (Table I). These mice each received 0·06 ml. 1/10,000 adrenaline (0·2 mg. per kg.) subcutaneously. In one, this was divided into two equal doses, separated by a 3 min. interval, and the mouse was frozen 3 min. after the second injection. In the other, the adrenaline was given in one dose, producing marked signs of stimulation, and the mouse was frozen 3 min. after injection. These experiments confirm the findings of Kerr *et al.* [1937].

In the tables, the experiments are numbered in the order in which they were carried out.

Table I. *Brain lactic acid in normal mice*

Exp. no.	Wt. of mouse g.	Sex	Brain lactic acid mg. per 100 g.	Remarks
			Without exercise	
1	25	F	23	—
2	24	F	25	—
10	24	M	16	—
15	20·5	F	23	—
18	28	F	16	—
23	21·5	M	19	—
30	25·5	M	16	14·5 hr. food deprivation
44	30·5	M	19	4 hr. food deprivation
61	20	M	12	3 hr. food deprivation
67	19	M	20	—
79	19·5	F	18	—
87	19·5	F	20	—
		Mean	18·9	
			After exercise	
5	21	M	25	Exercised 3 min.
9	21	M	27	Exercised 5 min.
14	23·5	F	27	Exercised 4 min.
19	32·5	F	27	Exercised 4 min.
27	21	M	28	Exercised 5 min.
		Mean	26·8	
			After Na lactate injection	
77	22	M	24	Injected 4 mg. lactic acid
78	26	F	14	Injected 4 mg. lactic acid
86	20·5	F	17	Injected 8 mg. lactic acid
		Mean	18·3	
			After adrenaline injection	
90	31·5	M	17	2 injections, 3 min. apart
91	26·5	M	16	1 injection

Effects of anaesthetics

Table II gives data obtained on mice killed in a state of anaesthesia. The experiments are arranged in the order of increasing duration of anaesthesia before killing, this period being measured from the time of loss of righting reflexes.

Table II. *Brain lactic acid in anaesthetized mice*

Exp. no.	Wt. of mouse g.	Sex	Induction period min.	Period of anaesthesia min.		Brain lactic acid mg. per 100 g.
Phenobarbital, excitement stage						
51	23·5	M	7	0		17
58	21·5	M	17	0		8
56	22	M	18	0		8
35	27	F	21	0		12
					Mean	11·2
Phenobarbital anaesthesia						
41	27	M	·15	15		9
49	26·5	M	30	18		8
48	25·5	M	13	22		8
34	24	F	30	42		11
36	18·5	M	30	70		6
50	22·5	M	25	196		14
57	24	M	20	210		6
					Mean	8·9
Amytal anaesthesia						
47	26	M	43	4*		9
46	29	M	10	15		8
45	23·5	F	10	23		8
43	26·5	F	10	25		7
					Mean	8·0
Ether anaesthesia						
55	24	M	1	0·5		16
81	23·5	M	1	1		18
76	24·5	F	3	5		8
80	20·5	F	1	10		12
71	23·5	F	3	15		13
72	25·5	M	0·5	15		11
75	23	M	3	20		9

* After a second injection.

The barbiturates were administered intravenously (tail vein), or in some cases subcutaneously, when the needle missed the vein. The manner of administration seemed to make little difference. Phenobarbital dosages were 0·04–0·06 ml. 10% phenobarbital as the Na salt per mouse. Amytal dosages were 0·1–0·15 ml. 5% Na amytal per mouse.

All three anaesthetics decreased the brain lactic acid significantly. With phenobarbital, it was shown that the decrease begins during the excitement stage. With ether, which induces anaesthesia much more quickly, the lactic acid decrease lags behind the onset of anaesthesia.

The lactic acid values obtained are lower than those reported by Kerr and coworkers for dog, cat and rabbit brains. However, it must be remembered that they analysed grey matter only, while the whole brain was used in the experiments reported here.

Effect of insulin

Table III gives the data obtained on mice killed in "insulin shock". The mice were deprived of food for periods of 3–16 hr. before the experiments. No correlation seemed to exist between the length of this period and the severity of symptoms or the brain lactic acid. The insulin used was 40 units per ml., Eli Lilly Company. Injections were into the tail veins, or occasionally subcutaneous.

In all mice killed during insulin convulsions, and in some killed while in a flaccid condition, the brain lactic acid was much lower than normal. This

Table III. *Brain lactic acid during "insulin shock" in mice*

Exp. no.	Wt. of mouse g.	Sex	Insulin injected units	Period of insulin action min.	Brain lactic acid mg. per 100 g.
		Killed while in flaccid condition			
25	25·5	M	1, 1, 2*	268	14
26	22	M	1	107	16
28	23·5	M	1, 1, 1*	240	2
68	20·5	M	2	35	6
		Killed during convulsions			
29	21·5	M	1	63	6
31	28·5	M	3	80	5
37	24·5	M	3	230	5
38	25	F	3	53	9
42	29	M	3	88	9
				Mean	6·8

* At intervals of 1–2 hr. Unsuccessful attempt to produce convulsions.

confirms the findings of Kerr *et al.* [1937], although earlier Kerr & Ghantus [1936] reported normal brain lactic acid values after insulin.

A low level of brain lactic acid during hypoglycaemia is in harmony with the view that the decreased glucose supply to the brain is an important factor in hypoglycaemic shock.

Effects of picrotoxin

Picrotoxin in large enough doses causes severe epileptiform convulsions in mice. In these experiments, the dosage used was 0·04–0·08 ml. per mouse, of a solution containing 3 mg. picrotoxin per ml. (in 9 % alcohol), Eli Lilly Company. The injections were made into the tail veins, or occasionally subcutaneously.

Table IV. *Mouse brain lactic acid in picrotoxin convulsions*

Exp. no.	Wt. of mouse g.	Sex	Duration of convulsions min.	Brain lactic acid mg. per 100 g.	Remarks
		Killed at beginning of convulsions			
6	21·5	M	—	26	—
7	23	M	—	19	—
12	22	M	—	24	—
20	27	F	—	13	—
21	23	F	—	15	—
24	25·5	M	—	27	—
32	30	M	—	18	—
33	25	M	—	12	—
39	20	F	—	18	—
40	24	F	—	13	—
			Mean	18·5	
		Killed during convulsions			
3	31·6	F	3	49	Very severe convulsion
4	18	F	8	36	Series of light convulsions
8	21	M	6	39	Last convulsion severe
11	32	M	2	27	All of brain except cerebellum
				30	Cerebellum only
13	22	F	7	30	Severe convulsion
16	20	M	5	34	—
17	33	F	10	37	Series of convulsions
22	21	M	6	42	Series of light convulsions
			Mean	36·9	

Convulsions invariably began in 5 to 8 min., being ushered in by a characteristic shaky appearance, hunching of the back and fighting movements of the front feet. In one mouse the convulsions terminated fatally after being allowed to continue for about 15 min.

In one group of experiments, the mice were frozen when the convulsion was just beginning, as judged by these characteristic signs, or immediately after it began. The brain lactic acid values were normal in this group, with a mean of 18·5 mg. per 100 g. (Table IV).

In a second group, the convulsions were allowed to continue for 2–10 min., and the mouse was then frozen during a convulsion. A significant increase in brain lactic acid occurred in this group, the mean value being 36·9 mg. per 100 g.

Kerr & Antaki [1937] reported experiments with picrotoxin, in which, however, the convulsion was terminated with an anaesthetic before the brain was frozen *in situ*. Using ether, they found a higher than normal brain lactic acid after picrotoxin, but in the case of amytal, which requires longer for induction of anaesthesia, and is pharmacologically antagonistic to picrotoxin, normal values were obtained.

Effect of metrazol

Metrazol convulsions were induced by injection of 0·05–0·07 ml. per mouse, of an aqueous solution containing 2 % metrazol (cardiazol) and 0·02 % Na_2HPO_4. The convulsant action was quite irregular. Sometimes a violent convulsion began within 2 sec., while in other cases it was delayed as much as 7 min., and frequently two or more injections were required. If the injection was subcutaneous, the onset was slower than if intravenous. The convulsions were always very brief, lasting only a few seconds. They sometimes ended fatally. Occasionally recovery occurred before the mouse could be frozen; in these cases a second convulsion sometimes occurred.

The brain lactic acid values obtained on mice frozen during metrazol convulsions varied over a wide range, from normal to very high values, with a mean of 26·1 mg. per 100 g. (Table V). The occurrence of some very high values is of importance, considering the very brief nature of these convulsions.

Table V. *Mouse brain lactic acid in metrazol convulsions*

Exp. no.	Wt. of mouse g.	Sex	Brain lactic acid mg. per 100 g.
52	26·5	M	33
53	22·5	M	25
54	26	F	29
59	21	M	20
60	20·5	M	20
62	25	M	27
63	26	M	21
64	26	M	39
65	22	M	29
66	19·5	M	18
		Mean	26·1

Kerr & Antaki [1937] found high brain lactic acid values after metrazol convulsions, even though amytal or ether was administered before freezing the brain *in situ*.

Effect of nicotine

Nicotine convulsions were produced by subcutaneous injections of 5–11 mg. nicotine per kg., in 1·5, 2·5, or 5 % solution. As with metrazol, the response was quite variable. In some cases repeated injections were made. When a convulsion

occurred, it began with co-ordinated running movements, 45 sec.–2 min. after the injection. These merged into the convulsion, which was usually very brief. Occasionally a series of light convulsions occurred. Recovery or death followed usually within 3 min., if the convulsion was allowed to continue.

The brain lactic acid values found on mice killed during nicotine convulsions ranged from 14 to 27 mg. per 100 g., with a mean of 23·2 mg. (Table VI). These results are not significantly different from those obtained on normal mice.

Table VI. *Mouse brain lactic acid in nicotine convulsions*

Exp. no.	Wt. of mouse g.	Sex	Brain lactic acid mg. per 100 g.
82	18·5	M	27
83	19·5	F	24
84	19·5	M	26
85	27·5	M	23
88	19	F	25
89	28	F	14
		Mean	23·2

Effect of NaCN

Cyanide convulsions were found to be difficult to produce. The response was quite variable, and frequently the mouse died without having a true convulsion. However, 4 mice were frozen during convulsions, after injection of 7–10 mg. NaCN per kg., by tail vein, in 0·2, 0·3 or 0·4% solution. In some, repeated injections were given, some of which may have been subcutaneous rather than intravenous. Probably all of these mice would have died very quickly if they had not been frozen.

The brain lactic acid values were very high, the mean being 62·8 mg. per 100 g. (Table VII).

Table VII. *Mouse brain lactic acid in cyanide convulsions*

Exp. no.	Wt. of mouse g.	Sex	Brain lactic acid mg. per 100 g.
69	22	F	82
70	24·5	F	56
73	21	M	51
74	22·5	F	62
		Mean	62·8

The data are in harmony with those of McGinty [1929], who found that impairment of brain oxidations by cyanide causes lactate to be given out to the blood by the brain.

DISCUSSION

The low brain lactic acid values found in anaesthetized mice are in accord with the view that the cerebral metabolism is depressed during anaesthesia. Dameshek *et al.* [1934] found that during amytal or ether anaesthesia a decrease occurs in the amounts of glucose and of O_2 removed from the blood by the brain. Quastel and his coworkers, as well as others, have observed that the O_2 consumption of brain tissue *in vitro* in the presence of certain substrates is depressed by anaesthetics [Quastel & Wheatley, 1932; 1934; Jowett, 1938].

However, the results reported here apparently do not support the hypothesis of Quastel, which attributes the anaesthetic action of the drugs to a specific inhibition of some part of an enzyme system involved in the oxidation of lactate or pyruvate. If this hypothesis were correct, one would expect to find an

accumulation of lactate in the brain during anaesthesia. It seems necessary to postulate some other effect of the anaesthetic on the brain tissue, besides the specific inhibition of oxidations observed by Quastel and coworkers. As to whether lactate formation from glucose is specifically inhibited by these anaesthetics, a search of the literature has revealed no data, although Loebel [1925] found that certain urethanes and higher alcohols inhibit respiration of brain tissue more than anaerobic glycolysis, and increase aerobic glycolysis. Inhibition of glycolysis would not explain all the observations of Quastel and coworkers.

The most obvious hypothesis to account for the variations observed in brain lactic acid content would be to suppose that the brain lactic acid content is dependent on the activity of the tissue, lagging somewhat behind the tissue activity, as in muscle. Thus, the brain lactic acid is low during anaesthesia, higher in the normal condition, still higher during exercise, which involves motor activity and highest during convulsions. McClendon [1912] has shown that tissue activity involves an increase in cell membrane permeability, and since intracellular metabolism and permeability are interrelated in some way as yet not understood, it is possible that certain metabolic changes are secondary to changes in membrane permeability. Such a hypothesis might also explain the observations of Quastel and coworkers. Spiegel & Spiegel-Adolph [1936] have shown that anaesthetics decrease the brain cell membrane permeability *in vivo*, confirming inferences drawn from observations on fish eggs by McClendon [1915] and on algae by Osterhout [1916].

The low lactic acid values occurring during insulin convulsions and during the excitement stage of phenobarbital anaesthesia do not accord well with the view that the brain lactic acid content is dependent on the tissue activity. The low values with insulin may be attributed to lack of the substrate, glucose, and in the case of phenobarbital, a "release" theory might be adopted, which would suppose that the higher centres are first anaesthetized, leaving the lower centres active during the excitement stage. The results with nicotine are also out of harmony with this first hypothesis, since the values are generally lower than those observed with metrazol, and none are out of the normal range.

A second hypothesis to account for the high values which occur during picrotoxin, and occasionally during metrazol convulsions, would be to suppose that these substances inhibit some part of an enzyme system involved in the brain oxidations, the accumulation of a measurable amount of extra lactate lagging somewhat behind the beginning of convulsions. In the case of cyanide convulsions, the symptoms are generally attributed to an inhibition of the cytochrome-indophenol oxidase system. Since the lactate accumulation was much greater with cyanide than with picrotoxin or metrazol, while the convulsions occurred with less regularity, it would be necessary to suppose that the inhibition of oxidation occurred at a different point in the oxidative mechanism. This is easily possible in a system so complex. The results with insulin are in harmony with this second hypothesis, since in hypoglycaemia the normal substrate for oxidation is lacking, and consequently the supply of oxidative energy required for the maintainance of homeostasis is deficient. The results with nicotine do not seem to be in accord with this hypothesis. Nicotine is of special interest in that it inhibits lactic dehydrogenase, increases anaerobic glycolysis, and decreases succinate oxidation by brain tissue *in vitro* [Himwich & Fazekas, 1935; Quastel & Wheatley, 1937]. Despite these reports, nicotine did not cause a significant increase of brain lactic acid *in vivo*.

That the extra lactic acid found in the brain during picrotoxin and metrazol convulsions does not come from the blood is shown by the normal or subnormal

values observed in conditions in which high blood lactic acid occurs, namely after lactate or adrenaline injection and during ether anaesthesia or insulin convulsions.

Finally, it is possible that the high brain lactic acid values occurring during picrotoxin or metrazol convulsions may be due to circulatory disturbances, either cerebral or systemic, resulting in cerebral anoxaemia. Cerebral vascular spasm is a possible cause of convulsions in some cases [Cobb, 1936]. In metrazol convulsions, Corrin [1938] observed temporary cardiac arrest in 10–15% of cases.

SUMMARY

1. A technique is described for determining the lactic acid content of the brain of the mouse, using liquid air and avoiding both post-mortem changes and the use of anaesthetics.

2. The lactic acid content of the brain of the normal mouse was found to vary from 12 to 25 mg. per 100 g. tissue, with a mean of 18·9 mg. per 100 g. This is increased to 26·8 mg. per 100 g. by strenuous exercise.

3. Anaesthetics (phenobarbital, amytal, ether) decrease the brain lactic acid content significantly. With phenobarbital the decrease begins during the excitement stage of anaesthesia; with ether it lags behind the induction of deep anaesthesia.

4. During insulin convulsions, the brain lactic acid is significantly decreased. During "insulin shock" without convulsions, a decrease is sometimes observed. This finding is attributed to the decrease in glucose supplied to the brain by the blood.

5. During picrotoxin convulsions which have progressed for 2–10 min., the brain lactic acid is significantly increased and is above the level observed in normal mice after strenuous exercise. At the beginning of the convulsion, no increase occurs above the level observed in normal resting mice.

6. During metrazol convulsions, the brain lactic acid varies from normal to significantly increased values.

7. During nicotine convulsions, no significant increase in brain lactic acid content was observed.

8. During cyanide convulsions, the brain lactic acid shows a large increase.

9. High brain lactic acid values cannot be attributed to diffusion of lactate from the blood into the brain.

10. Possible interpretations of the data, and their significance in relation to theories of anaesthesia and to the causation of convulsions are discussed.

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CCXLVII. ENZYMIC PROTEOLYSIS

II. THE FORMATION OF FREE TYROSINE IN PEPTIC DIGESTION

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THE liberation of free amino-acids during the digestion of proteins by pepsin has recently become the subject of controversy [cf. Abderhalden, 1937]. The view generally held has been that the action of pepsin ends with the fission of proteins into high-molecular complexes from which amino-acids are set free only by the further action of enzymes with specific peptidase function. In considering the evidence for this view it is necessary to bear in mind that most of the experiments on which it is based were carried out more than 30 years ago [Abderhalden, Baumann, Kautzsch, Körösy & London, 1905–7] and were designed mainly to throw light on the physiological question of the fate of protein in the alimentary tract. The procedure adopted in the well-known experiments of Abderhalden, London and co-workers cited above was to analyse the intestinal contents of dogs collected soon after a protein meal either by killing the animals or by means of fistulae established at different points of the alimentary tract. The experiments led to valuable results on which much of our present knowledge of digestion and absorption of proteins is based, but the chemical technique available was hardly adequate to elucidate the finer chemical differences in the mode of action of the proteolytic enzymes concerned. Thus in one paper by Abderhalden *et al.* [1906] we have the interesting statement, "Eine quantitative Methode zur Bestimmung von Aminosäuren aus Gemischen kennen wir nicht". Distillation after esterification was the method adopted for the detection of amino-acids in the mixtures they were dealing with. From stomach brei, in contrast to material obtained from the duodenum, jejunum etc., amino-acid esters were obtained either not at all, or only in traces. In view of the fact that esterification itself involved processes that might cause protein hydrolysis, it is not surprising that Abderhalden [1931] came to the conclusion that peptic digestion differs from that by trypsin and erepsin in not leading to the formation of free amino-acids.

More recently the same conclusion has been reached by Waldschmidt-Leitz & Künstner [1927] on less justifiable grounds. These authors used alcoholic titration for studying the extent of proteolysis of thymus histone and, on the assumption that peptides in contradistinction to amino-acids titrate quantitatively in 50% alcohol (for which we have found no theoretical or practical justification [cf. Harris, 1929]), claim to have proved the absence of amino-acids from peptic digests.

The evidence for the contrary view rests upon the findings of Northrop [1930] and Calvery & Schock [1936]. The earlier claim of Felix [1925] to have isolated lysine from peptic digests of thymus histone (as benzoyllysine) cannot be accepted without further confirmation, for, according to this author the liberation of free lysine was unaccompanied by any increase in amino or carboxyl groups and involves the assumption that the combination of lysine in histone is different

from that in other proteins. Northrop [1930] found that during the auto-digestion of crystalline pepsin "a large amount of tyrosine crystallised out". From 100 g. of egg albumin digested with 20 g. of pepsin for 26 days Calvery & Schock [1936] isolated 565 mg. of tyrosine; from the same amount of pepsin allowed to digest itself only 150 mg. of tyrosine were obtained.

Abderhalden [1937], on repeating these experiments with casein, was unable to obtain any tyrosine by direct crystallization and ascribes Calvery's findings to bacterial contamination, which according to the former is possible even in the strongly acid medium required for peptic digestion. Exception has been taken to these results also on the ground that the tyrosine present might have been formed through the agency of the acid present in the digest [Oppenheimer, 1936].

We have frequently noticed the crystallization of small quantities of tyrosine in concentrating large scale peptic digests of proteins prepared for various researches. But at the same time the validity of the objections raised by Abderhalden and Oppenheimer could not be denied as the digests were usually allowed to stand for varying periods and no special precautions against bacterial contamination had been taken. Calvery also allowed a digestion period of 26 days and the solution from which he obtained his tyrosine had been kept for 4 months in the ice chest. Digestion experiments were therefore carried out with casein, taking all precautions for excluding bacterial action, with proper controls to take into account the possible formation of tyrosine by the action of the acid medium or by the self-digestion of the enzyme employed, and limiting enzyme action to a short period. From a number of such digests, after preliminary treatment with phosphotungstic acid, we have been able to crystallize out appreciable quantities of tyrosine without any difficulty.

Quantitative determinations were also made during the course of digestion of the extent of peptide groups hydrolysed by formaldehyde titration and of the amount of tyrosine not precipitable by phosphotungstic acid by the colorimetric method of Folin & Ciocalteu [1927]. It is perhaps necessary to make it clear that the latter method does not determine free as distinct from combined tyrosine. Being based upon Millon's reaction which requires only the phenolic hydroxyl of tyrosine to be uncombined [Vaubel, 1900] the determination is valid for tyrosine in solution whether free or in peptide combination, though the fact that it is generally applied to hydrolysed protein has probably caused some misunderstanding. In a method due to Zuwerkalow [1927], of which the Folin-Ciocalteu method is a modification, the determination is made on the intact protein and we have found that satisfactory values of the tyrosine content of proteins can be obtained by this procedure. Emphasis has been laid on this point because in similar experiments on peptic digests analytical values meant to show that arginine was present in the form of low-molecular compounds not precipitable by salicylsulphonic acid [Lieben & Lieber, 1934] have been mis-construed [Calvery et al., 1936] to mean the presence of free arginine.

In the present experiments the tyrosine values were determined after different periods of digestion on the filtrates after treatment of the digest with phosphotungstic acid in H_2SO_4; these values, therefore, represent the amount of tyrosine not combined in the form of large polypeptides (which can justifiably be assumed to be precipitable by phosphotungstic acid) and serve the purpose of setting the upper limit to the amount of free tyrosine present at any stage of digestion. The values given in Table I show the progress of peptide hydrolysis and the liberation of tyrosine, in so far as the latter is indicated by the amount present in the phosphotungstic acid filtrate, in a typical experiment. At the

end of 7 days' digestion with pepsin 6·13% of the tyrosine in casein was in a form not precipitable by phosphotungstic acid. Analysis of the digest at this stage also showed that the filtrate from phosphotungstic acid contained 6·02% of the total N of the digest, of which 45% was in peptide form (Table II).

The yield of pure tyrosine isolated from one such digest was 240 mg. from 203·3 g. of casein, equivalent to 1·8% of the total tyrosine present in the casein or 61% of the tyrosine not precipitable by phosphotungstic acid. From the mother liquor from the first crop of tyrosine a second solid fraction weighing 342 mg. could be obtained by further evaporation which, according to colorimetric estimation, contained 103 mg. of tyrosine; but the pure amino-acid could not be isolated from this fraction by recrystallization and it is not possible to say if this tyrosine was present in the free or combined state. In a control experiment without the addition of pepsin, in which an equal weight of casein was kept under otherwise identical conditions of temperature and acidity for a period of 14 days, not only could no tyrosine be isolated but the phosphotungstic acid filtrate did not show the slightest trace of tyrosine free or combined on being concentrated and treated with Millon's reagent. In an enzyme control in which an equal weight of pepsin was allowed to digest itself the phosphotungstic acid filtrate contained less than 17 mg. of tyrosine.

Abderhalden, in contradicting the findings of Calvery, has laid emphasis on the possibility of micro-organisms being active in peptic digests in spite of the acidity of the medium, as lumps of protein are usually present during the initial stages and the interiors of these would not be accessible to the bactericidal action of the acid. In one experiment, therefore, casein suspended in water was first sterilized by heat before the addition of H_2SO_4 and pepsin. From 202·5 g. of sterilized casein digested for 14 days 234 mg. of pure tyrosine and a further 145 mg. of the nearly pure amino-acid were isolated. From sterilized casein without the addition of pepsin, but kept under otherwise identical conditions, no tyrosine could be detected in solution.

There can be no doubt, therefore, that peptic digestion gives rise to free tyrosine. The amount of free amino-acids produced by the action of pepsin may be too small to be of physiological significance on account of the short time during which the chyme remains in the stomach, but in studying the chemical mode of action of the enzyme and its relation to protein structure, the ability of pepsin to liberate amino-acids will undoubtedly have to be taken into account.

EXPERIMENTAL

Rate of liberation of tyrosine

50 g. casein were digested with 2·5 g. pepsin under conditions described later in the section on isolation. Peptide hydrolysis was determined daily on 5 ml. aliquots by formaldehyde titration. Simultaneously, 100 ml. of the digest were pipetted out for determination of tyrosine not precipitable by phosphotungstic acid by the following procedure: the solution was made 5% acidic with 50% H_2SO_4 and treated with a 30% solution of phosphotungstic acid in 5% H_2SO_4 added in slight excess. The precipitate was separated by centrifuging and washed thoroughly with dilute phosphotungstic acid-H_2SO_4. The centrifugate and washings were combined, freed from phosphotungstic acid and H_2SO_4 by baryta which was itself quantitatively eliminated by H_2SO_4, concentrated *in vacuo* at 40° and finally made up to 25 ml. Tyrosine was estimated on 10 ml. aliquots according to Folin & Ciocalteu [1927]. Total tyrosine in the digest was determined by the method of Zuwerkalow [1927].

The extent of peptide hydrolysis at each stage was calculated from the increase in amino-N as determined by formaldehyde titration, by comparison with the amino-N on complete hydrolysis. The value of the latter was taken to be 68·14% of the total N as calculated from Van Slyke's data for the N distribution of casein [Plimmer,1917] by adding together the whole of the amino-N, $\frac{1}{4}$ of the arginine-N, $\frac{1}{3}$ of the histidine-N and the whole of the lysine- and cystine-N; the total N in the solution was estimated in a 5 ml. aliquot by the micro-Kjeldahl method.

The results are summarized in Table I.

Table I

50 g. B.D.H. "Light White" casein + 2·5 g. B.D.H. pepsin in 1 litre approximately $N/20$ H_2SO_4, pH 1·8.

Total N	= 30·07 mg./5 ml.	
Amino-N on complete hydrolysis (calc.)	= 20·48 mg./5 ml.	
	= 14·63 ml. $N/10$ NaOH	
Total tyrosine	= 261 mg./100 ml.	

Days	Formaldehyde titre; ml. $N/10$ NaOH	Peptide hydrolysis %	Tyrosine in phosphotungstic acid filtrate	
			mg./100 ml. of digest	% of total tyrosine
0	0·83	—	4·41	1·69
1	1·97	7·79	4·41	1·69
2	2·43	10·93	6·37	2·44
3	3·12	15·65	7·93	3·04
4	3·44	17·83	12·00	4·60
5	3·53	18·45	13·22	5·07
6	3·62	19·06	15·73	6·03
7	3·70	19·62	16·00	6·13

Composition of the phosphotungstic acid filtrate at the end of digestion

On the 7th day 200 ml. of the digest were pipetted out and treated with phosphotungstic acid as already described. After removal of reagents the solution was concentrated *in vacuo* and made up to 25 ml. Total N (micro-Kjeldahl) and amino-N (Van Slyke) were determined on aliquots. 10 ml. of the solution were hydrolysed with 30 ml. of conc. HCl for 10 hr.; the HCl was removed as completely as possible by distillation to dryness *in vacuo*, the solution made faintly alkaline by adding a 10% suspension of baryta and distilled again under reduced pressure to expel any ammonia present. The residual solution was made up to 25 ml. and amino-N estimated in aliquots. The results are given in Table II.

Table II

	mg./200 ml. digest	%
Total N in digest	1202·6	—
Total N in phosphotungstic acid filtrate	72·4	6·02
Amino-N in phosphotungstic acid filtrate before hydrolysis	37·68	52·05
Amino-N in phosphotungstic acid filtrate after hydrolysis	70·55	97·45
Peptide-N	—	45·40

Isolation of tyrosine

250 g. of B.D.H. light white casein were ground up to a uniform suspension with 2·5 l. of $N/10$ H_2SO_4 at 37° and mixed with an equal volume of sterile water in a sterilized pyrex flask. After adjusting to pH 1·8, 7·5 g. of B.D.H. pepsin dissolved in a small quantity of $N/20$ H_2SO_4 were added and the mixture incubated

at 37°. Care was taken to ensure that there were no particles of protein adhering to the sides of the flask above the liquid level and sufficient toluene was added to form a thick layer reaching to the neck of the flask. After a day's digestion the pH, which had changed to 2·2, was readjusted and a further 5 g. of pepsin dissolved in 25 ml. of $N/20$ H_2SO_4 added. The pH showed no appreciable change after the third day. After the first few days the "paranuclein" separating out of the solution settled to the bottom, giving a clear golden brown supernatant liquid.

After 7 days, when formaldehyde titration showed a hydrolysis of 18·01 %, 3800 ml. of the digest (containing on the basis of N determinations 203·3 g. of casein) were filtered, the clear solution cooled in ice and treated with sufficient 50% H_2SO_4 also previously cooled in ice, to make the whole solution 5% acidic with respect to H_2SO_4. A concentrated solution of phosphotungstic acid in 5% H_2SO_4 was now added with vigorous mechanical stirring until precipitation was complete, the temperature being maintained throughout at 0°. About 600 g. phosphotungstic acid were required. The mixture was left in the ice chest for 2 days after which the bulky precipitate was removed by centrifuging and washed with ice-cold dilute phosphotungstic acid. To the combined solution and washings baryta was added in the form of a thin cream. The precipitate of barium phosphotungstate and sulphate was separated on the centrifuge and washed thoroughly with baryta water. The combined supernatant liquids were then freed from Ba quantitatively by means of H_2SO_4. The final solution, which was about 5 l. in volume and quite colourless, was concentrated under reduced pressure at 45° to about 700 ml. The solution, which at this stage had a pale bluish tinge, was again made strongly alkaline with baryta to precipitate residual phosphotungstic acid, the small precipitate formed centrifuged off and the Ba again quantitatively removed from the solution by dilute H_2SO_4. The solution was again concentrated *in vacuo* to about 125 ml. and then transferred to a crystallizing dish in a vacuum desiccator over H_2SO_4. Characteristic crystals of tyrosine separated overnight. They were filtered with suction, washed with a little ice-cold water, then with alcohol and ether and finally dried in the vacuum desiccator over H_2SO_4. The solid was perfectly white and weighed 243 mg.

A specimen dried *in vacuo* over P_2O_5 at 100° gave on analysis N, 7·597%, amino-N, 7·602%. $C_9H_{11}NO_3$ requires N, 7·735%. After a single recrystallization from hot water, without the use of charcoal, the values were N, 7·716%, amino-N, 7·732%.

The mother liquor on further concentration in the vacuum desiccator gave a thick deposit of solids which was filtered on a glass filter with suction. The material was inclined to be sticky but was obtained as a granular powder by repeated washing with 95% alcohol. It weighed 342 mg. and contained 9·605% N and 9·171% amino-N. Pure tyrosine could not be isolated from this fraction by recrystallization, but colorimetric estimation showed that it contained 103 mg. of the amino-acid. A trace of the solid gave a violet coloration with quinone in the cold in the presence of a drop of sodium carbonate.

The alcoholic washings from the second crop of solids on evaporation to dryness gave a thick syrup which contained 26·05 mg. total N and 13·02 mg. amino-N indicating the presence of peptides. Tests for tryptophan were negative; Millon's reagent gave a feeble colour.

Substrate control

250 g. of casein were suspended in 5 l. of $N/20$ H_2SO_4 and, after adjustment of the pH to 1·8, left in the incubator under toluene. After 14 days the clear liquid was filtered from the unaltered casein and treated with phosphotungstic acid as already described. The slight precipitate that was obtained was filtered off and the filtrate freed from reagents. The final solution, having a volume of about 5 l., gave no colour with Millon's reagent. It was concentrated under reduced pressure to about 500 ml. At this stage also Millon's reaction was negative. The last traces of H_2SO_4 were now quantitatively removed and the solution concentrated to about 10 ml. when a minute amount of crystalline material separated out, the Millon's reaction being, however, still negative. The separated crystals were inorganic.

Enzyme control

2·5 g. of pepsin were dissolved in 1 l. of $N/20$ H_2SO_4 (same concentration of enzyme as used in the digests) and incubated at 37° under toluene. After 7 days 200 ml. of the digest were pipetted out and precipitated with phosphotungstic acid in the usual way. The filtrate, after removal of phosphotungstic acid, etc.. was concentrated *in vacuo* and made up to 25 ml. Aliquots of 10 ml. were taken for the duplicate estimation of tyrosine by Folin & Ciocalteu's method. Since the amount of tyrosine contained in the solution was small a known volume of standard tyrosine solution was added to the unknown before making colour comparison and the amount originally present obtained by difference. 200 ml. of the enzyme solution were found to contain 0·692 mg. of tyrosine not precipitable by phosphotungstic acid.

Isolation of tyrosine from sterilized casein digest

Casein prepared according to Cohn & Hendry [1930] was used directly after the final precipitation and washing without drying. Sufficient wet casein to give about 250 g. dry protein was suspended in 2·5 l. of water and sterilized by heating in a steamer for 1 hr. each day on three successive days. To the suspension cooled to 37° 2·5 l. of approximately $N/10$ H_2SO_4 were added slowly and the pH adjusted to 1·8. With vigorous mechanical stirring during the addition of acid formation of lumps could be completely avoided. 12·5 g. of pepsin in dilute H_2SO_4 were added and the mixture incubated under a thick layer of toluene. To avoid all chances of bacterial contamination no samples were withdrawn until the end of the digestion period of 14 days. The procedure after this stage was exactly as described before.

From 4·5 l. of the digest which, according to analysis, contained 1·32 g. of tyrosine on the whole and 517 mg. in a form not precipitable by phosphotungstic acid, two crops of crystals weighing 234 mg. and 145 mg. respectively were isolated. The first fraction gave N, 7·36% and amino-N, 7·49% on isolation and 7·67% and 7·65% respectively after one recrystallization. The second fraction which was not recrystallized gave N, 7·85% and amino-N, 7·97%. The result with sterilized casein suspended in acid without pepsin was exactly the same as with the substrate control already described.

SUMMARY

The liberation of tyrosine during peptic digestion of casein has been quantitatively followed. It is shown that after 7 days' digestion about 6% of the total tyrosine and roughly the same proportion of the total N of the protein are in a form not precipitable by phosphotungstic acid.

From peptic digests of casein, carried out under conditions where bacterial contamination was completely excluded, tyrosine has been isolated in yields amounting to 1·7–1·8% of the total tyrosine in the protein.

The tyrosine produced by self-digestion of the enzyme forms but a small fraction of that obtained from the casein digest.

Action of acid in the concentration necessary for peptic digestion does not lead to the liberation of the free amino-acid from casein.

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CCXLVIII. THE NON-SPECIFICITY OF THE ASCORBIC ACID OXIDASE

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THE observation that the retention of *l*-ascorbic acid and its analogues in the body of the guinea-pig was controlled by their stereochemical structure as well as by their antiscorbutic potencies [Zilva, 1935, 1] suggested that the extension of this work to the investigation of the bearing of the spatial arrangement of the molecule on other biochemical reactions might be advisable. It was considered that the advance of the general problem of the mechanism of the biological action of *l*-ascorbic acid in the animal organism might be furthered by this means.

With this end in view attention was directed to the reversible oxidation of *l*-ascorbic acid and its related compounds by enzymes. In a series of investigations the following results emerged. The enzyme first described by Szent-Györgyi [1930; 1931], which oxidizes *in vitro* *l*-ascorbic acid but not phenols, also oxidized the analogues of ascorbic acid. In addition these compounds could be oxidized by the phenolases present in the apple or potato but only in the presence of mono- or polyhydric phenols or the juice of the plants [Zilva, 1934; 1935, 2; Johnson & Zilva, 1937, 1]. From this it was inferred that the dehydrogenation was brought about by quinones formed by the action of the phenolases on the above substrates. Keilin & Mann [1938] have since found that this also holds true of the polyphenol oxidase present in the cultivated mushroom. This enzyme system which is evidently distinct from the ascorbic acid oxidase apparently functions also *in vivo*, since it has been observed that the equilibrium between *l*-ascorbic acid and dehydroascorbic acid in the living tissue of the apple changes progressively with the development of the fruit [Zilva *et al.* 1935; 1938]. Although the ascorbic acid oxidase oxidized all the analogues of *l*-ascorbic acid investigated, the rate of oxidation was markedly influenced by the stereochemical structure of these compounds. In fact a certain parallelism existed between the influence of structure on their rate of oxidation on the one hand and their capacity of being retained in the animal organism on the other [Johnson & Zilva, 1937, 2]. Thus the compounds in which the oxygen ring engages the hydroxyl group to the right of the carbon chain and which invariably possess antiscorbutic potency were oxidized at a much higher rate than their antiscorbutically inactive enantiomorphs. In contradistinction to this the asymmetry of carbon 5 or the presence of a seventh carbon atom in the chain which conditions the intensity of the antiscorbutic activity of the compounds with the ring to the left of the carbon chain had no influence upon the kinetics of the enzymic oxidation.

It remained to ascertain whether the activity of the ascorbic acid oxidase was restricted to ascorbic acid and its analogues or whether it extended to other compounds capable of reducing indophenol. Preliminary experiments with reductone and dihydroxymaleic acid suggested that these substrates may also be oxidized by it [Johnson & Zilva, 1937, 1]. These results, however, were not

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sufficiently clear-cut to justify a final conclusion. Having overcome the technical difficulties first encountered it was possible to investigate the problem of the specificity of the enzyme in greater detail. The results obtained with substrates of different chemical constitution form the subject of this investigation.

Technique

The oxidation of the compounds was assessed by means of a Barcroft-Warburg respirometer and the general technique was similar to that already described [Johnson & Zilva, 1937, 2]. The experiments were carried out at 26° and at pH 6·0. The pH at the end of the experiment never varied by more than 0·2. The volume of the oxygen was calculated for 760 mm. pressure. As a source of enzyme cucumber juice was employed, prepared by freezing the entire cucumber at −20° and pressing out the thawed pericarp. Some extraneous matter was then removed by adjusting the pH of the juice to 7·6–8·0, filtering and acidifying to its original pH (approximately 6·0). This enzyme preparation was dialysed in cellophane bags at 1° for 2 days against running distilled water. The quantity of enzyme which absorbed 10 μl. of O_2/min. in oxidizing 2·5 ml. 0·01 N l-ascorbic acid at 760 mm. pressure, 26° and pH 6·0 (M/15 phosphate buffer) was arbitrarily adopted to represent 10 units. The enzyme was always diluted to 1 ml. and the substrate in the buffer to 1·5 ml. Control experiments were performed in every case.

RESULTS

Reductic acid as substrate

The reductic acid [Reichstein & Oppenauer, 1933; 1934] was a sublimed and recrystallized sample kindly placed at our disposal by Prof. T. Reichstein. It was found by us to be 99–100% pure by indophenol titration.
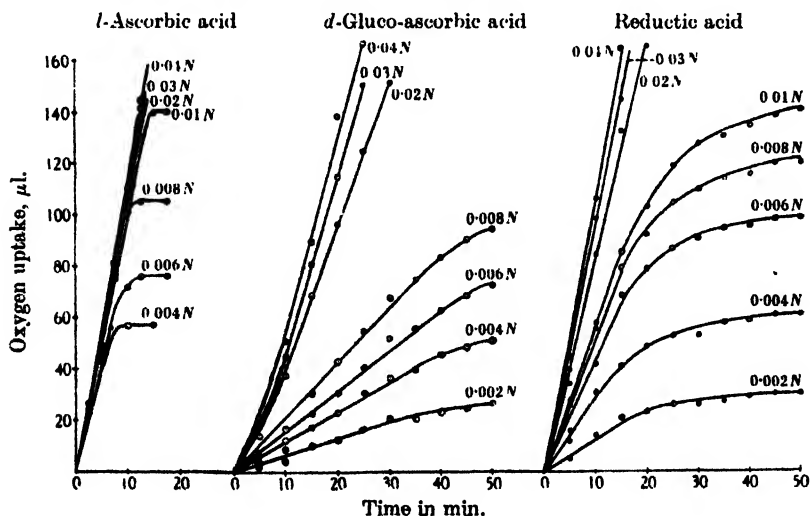
Fig. 1. Rates of oxygen uptake by l-ascorbic acid, d-gluco-ascorbic acid and reductic acid at varying concentrations expressed in normality. Strength of enzyme (undialysed), 10 units.

The actions of dialysed and undialysed enzyme were studied on this substrate. Since, apart from a difference in the relative rates, the oxidations proceeded similarly, the results obtained with undialysed juice only will be given. Fig. 1

represents the course of oxidation of varying concentrations of l-ascorbic acid, d-gluco-ascorbic acid and reductic acid in the presence of 10 units of the enzyme.

It will be seen that the rate of oxidation of reductic acid, like that of d-gluco-ascorbic acid, fell off after a time and was not linear as was found with l-ascorbic acid. This falling off was even more noticeable when dialysed juice was used, especially at high substrate concentrations.

In Fig. 2 the oxygen absorbed per min. during the initial stages, represented by linear portions of the graphs in Fig. 1, was plotted against concentrations of substrate. From this it may be observed that for low concentrations an increase of substrate raised the rate of oxidation of l-ascorbic acid much more than that of the other two compounds. With higher concentrations, however, such increase had hardly any perceptible effect on the oxidation velocity in the case of the former, but a considerable, although reduced, effect on that of the latter. This phenomenon may possibly be due to a lower combining affinity of the enzyme for d-gluco-ascorbic acid and reductic acid.

The results obtained with varying quantities of enzyme and constant concentrations of substrate (0·01 N) are given in Figs. 3 and 4. As was to be expected the rate of oxidation was found to be almost directly proportional to the enzyme activity. From Fig. 4 the higher oxidation velocity for l-ascorbic acid emerges once more.
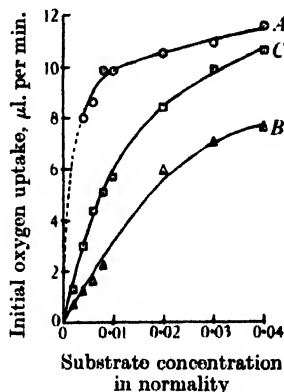
Fig. 2. Initial rates of oxygen uptake by l-ascorbic acid, d-gluco-ascorbic acid and reductic acid plotted against substrate concentration. Derived from Fig. 1. $(\cdot)—\odot$ (A). l-ascorbic acid. $\triangle—\triangle$ (B) d-gluco-ascorbic acid. $\square—\square$ (C) reductic acid.
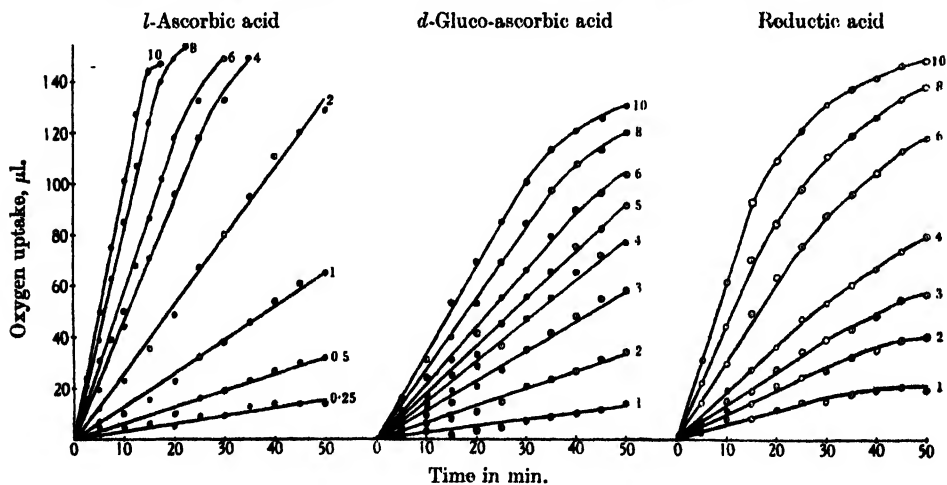
Fig. 3. Rates of oxygen uptake by l-ascorbic acid, d-gluco-ascorbic acid and reductic acid at varying enzyme concentrations expressed in units. Substrate concentrations, 0·01 N. Undialysed juice used throughout.

In order to ascertain whether the amount of oxygen absorbed was equivalent to the amount of substrate destroyed, the following experiment was devised. Out of a battery of eight Warburg flasks treated in the usual manner, each containing the same amount of enzyme and substrate, one was reserved for

manometric readings; the remaining seven were removed singly at suitable intervals and immediately titrated with $N/1000$ indophenol. The results were calculated on the assumption that 1 mol. of each of the substrates required 1 atom of oxygen and the values obtained were corrected to 760 mm. pressure
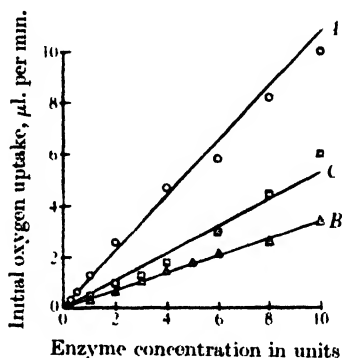
Fig. 4. Initial rates of oxygen uptake by l-ascorbic acid, d-gluco-ascorbic acid and reductic acid plotted against enzyme concentration. Derived from Fig. 3. ☉—☉ (A) l-ascorbic acid. ▲—△ (B) d-gluco-ascorbic acid. ☐—☐ (C) reductic acid.
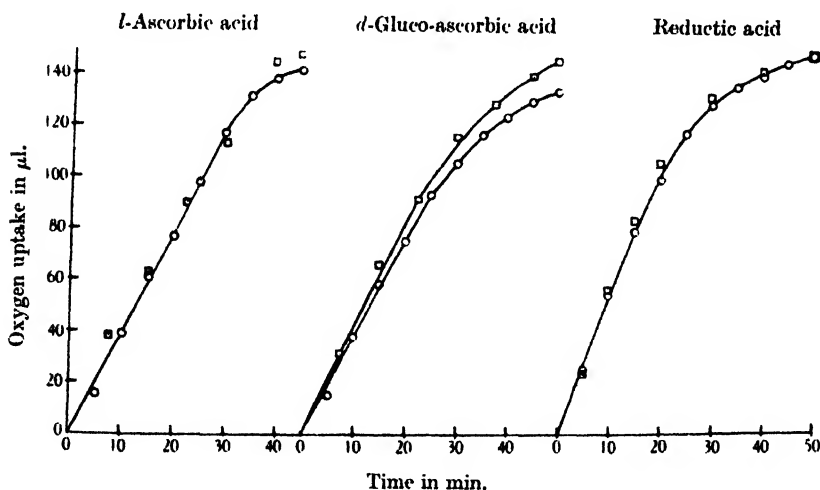
Fig. 5. Comparison of observed oxygen uptakes by l-ascorbic acid, d-gluco-ascorbic acid and reductic acid in the presence of undialysed cucumber juice, with that calculated from the disappearance of the substrates. ☉—☉ observed values. ☐—☐ calculated values.

and 26°. It will be seen from Fig. 5 that these figures agree well with those observed simultaneously by direct manometric measurements. We do not consider the slight deviation between the d-gluco-ascorbic acid graphs to be significant.

Reductone as substrate

Reductone was prepared by the method of Euler & Klussmann [1933] and Euler & Martius [1933, 1, 2, 3, 4]. The crude material was purified from hot ethyl acetate and then crystallized from butyl alcohol. The slightly brown product obtained in this way did not give a distinct M.P. (charring above 200°) thus resembling the product obtained by Euler & Martius. Further purification

revealed that this material contains a hitherto unsuspected impurity although by indophenol titration it was found to be 97% pure. All the experiments described below were therefore carried out with a preparation which was further purified by sublimation under low pressure at 125°. Two such treatments removed all the non-volatile impurities which remained as a feathery brown residue present to the extent of 8–10% of the crystallized product. The sublimed reductone, colourless needles, melted at 154–156° (decomp. uncorr.) according to the rate of heating. It must be pointed out that anomalous results were obtained when the non-sublimed substance was used as substrate.

Reductone reduces indophenol much more slowly than ascorbic acid and its analogues. It is, however, possible to obtain a definite end point if the titration is carried out at pH 4·5 and the indicator added slowly. Sublimed reductone was found to be 98–99% pure by this titration method.
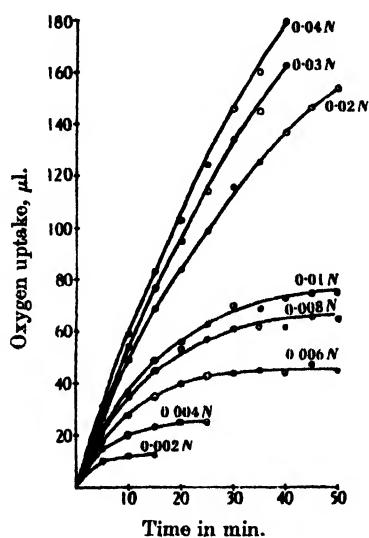
Fig. 6.
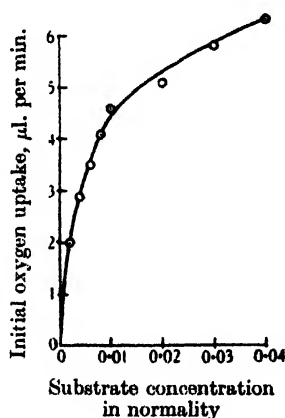
Fig. 7.

Fig. 6. Rate of oxygen uptake by reductone at varying concentrations expressed in normality. Strength of enzyme (undialysed juice), 10 units.

Fig. 7. Initial rate of oxygen uptake by reductone plotted against substrate concentration. Derived from Fig. 6.

As with d-gluco-ascorbic acid and reductic acid there was some inhibition as the oxidation of the reductone proceeded (Figs. 1 and 6). The increase in the initial velocity corresponding to the linear portions of the curves (Figs. 2 and 7) with the increase in substrate follows in this graph a form similar to that observed in the d-gluco-ascorbic acid and reductic acid graphs. These results which were obtained with undialysed juice were similar to those observed when the dialysed preparation was employed.

In the case of the undialysed juice, when the quantity of enzyme was varied and the concentration of the substrate (0·01 N) was kept constant the increase in enzyme at low concentrations brought about a very marked acceleration of the rate of oxidation. As the quantity of enzyme added was increased, however, a stage was reached when a further increase in the enzyme brought about

remarkably little response (Figs. 8 and 9). When dialysed juice was used the rate of O_2 uptake at low enzyme concentrations was greatly reduced (Fig. 10). The curve obtained by plotting initial velocities of O_2 uptake against enzyme

Time in min.

Fig. 8.

Enzyme concentration in units (undialysed juice)
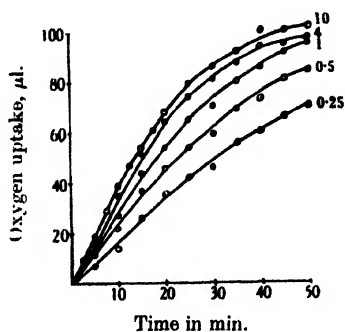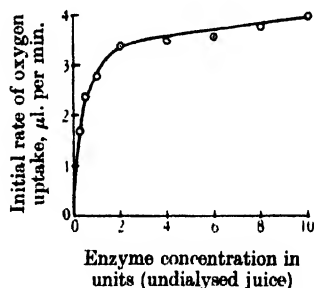
Fig. 9.

Fig. 8. Rate of oxygen uptake by reductone in the presence of varying amounts of *undialysed* cucumber juice. Enzyme concentrations expressed in units. Substrate concentration = $0.01 N$.

Fig. 9. Initial rate of oxygen uptake by reductone plotted against enzyme concentration. Derived from Fig. 8 and containing some points corresponding to curves which could not conveniently be included in Fig. 8.

Time in min.

Fig. 10.

Enzyme concentration in units (dialysed juice)

Fig. 11.

Time in min.
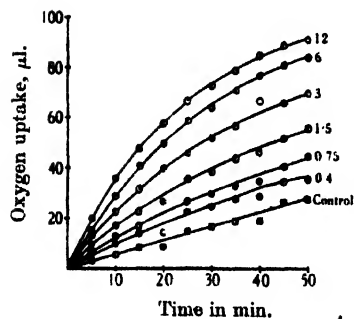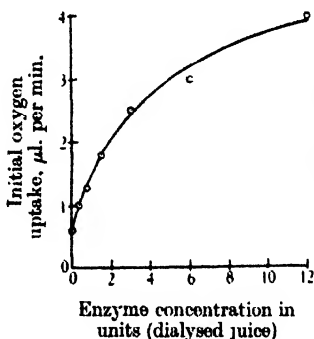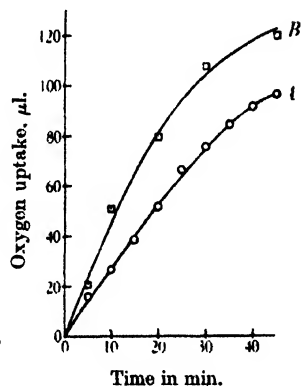
Fig. 12.

Fig. 10. Rate of oxygen uptake by reductone in the presence of varying amounts of *dialysed* cucumber juice. Enzyme concentrations expressed in units. Substrate concentration. $0.01 N$.

Fig. 11. Initial rate of oxygen uptake by reductone plotted against enzyme concentration. Derived from Fig. 10.

Fig. 12. Comparison of observed oxygen uptake by reductone in the presence of undialysed cucumber juice with that calculated from the disappearance of the substrate. ⊙—⊙ (*A*) observed values. ▫—▫ (*B*) calculated values.

concentration (Fig. 11), however, shows that this relationship is again not linear. This behaviour of reductone therefore stands out in striking contrast to that of the other compounds (Figs. 3 and 4).

From Fig. 12 it is seen that the disappearance of the substrate as determined by the method described above was greater than would have been expected

from the amount of O_2 absorbed. The difference is significant since it was found to be reproducible. In this experiment undialysed juice was used but similar results were obtained also with dialysed juice.

Dihydroxymaleic acid as substrate

A commercial sample of dihydroxymaleic acid was purified by recrystallization at 60° from butyl alcohol. The scintillating white crystalline product became powdery on drying *in vacuo* over conc. H_2SO_4. As dihydroxymaleic acid is not very soluble in water it was found convenient to employ its Na salt which was prepared by treating the acid in ethyl alcohol with 2 mol. of sodium ethoxide. The precipitated salt was washed with ethyl alcohol and dried.

The oxidation of dihydroxymaleic acid is characterized by the formation of CO_2, and in order to obviate interference with the measurements of the O_2 uptake the former was absorbed by placing 0·3 ml. of 20 % KOH solution and a roll of filter paper in the central cup of each manometer flask.
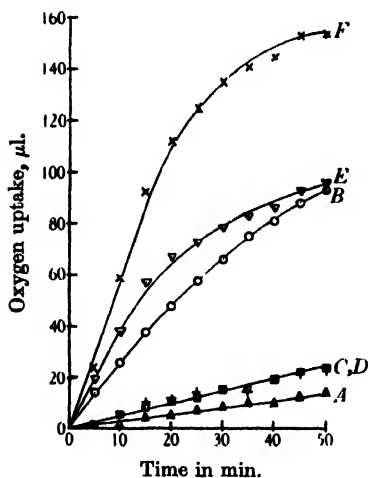
Fig. 13.
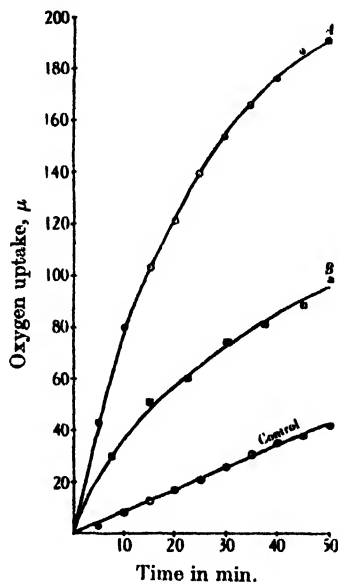
Fig. 14.

Fig. 13. Oxidation of $0·01 N$ dihydroxymaleic acid solutions. △—△ (A) with no addition (control). ⊙—⊙ (B) in the presence of 1 ml. undialysed cucumber juice. ⊡—⊡ (C) in the presence of 1 ml. dialysed cucumber juice. +—+ (D) in the presence of 1 ml. dialysed cucumber juice containing 10 mg. *l*-ascorbic acid per 100 ml. ▽—▽ (E) in the presence of 1 ml. dialysed cucumber juice and $M/100$ phenol. ×—× (F) in the presence of 1 ml. dialysed cucumber juice and $M/100$ catechol.

Fig. 14. Comparison of observed oxygen uptake by dihydroxymaleic acid in the presence of undialysed cucumber juice with that calculated from the disappearance of substrate. ⊙—⊙ (A) observed values. ⊡—⊡ (B) calculated values.

The action of cucumber juice upon dihydroxymaleic acid solutions differed from that on the preceding substrates in being greatly influenced by dialysis. Whilst the untreated juice caused a considerable O_2 uptake, after dialysis the uptake was hardly more than in the control. Further experiments showed that addition of quantities of ascorbic acid up to 10 mg. per 100 ml. of dialysed juice did not restore its catalytic activity towards dihydroxymaleic acid. On the

other hand, when phenol or catechol was added to the dialysed preparation, so that the final mixtures in the flasks were $M/100$ in respect to these compounds, considerable activity was imparted (Fig. 13).

It is of interest to note that there was a great discrepancy between the volume of O_2 calculated from the disappearance of the substrate in the presence of undialysed juice and the volume of O_2 actually taken up (Fig. 14). This fact, taken in conjunction with the observation made by us that CO_2 was evolved in the process, suggests that the mechanism involved in this oxidation was undoubtedly different from that associated with the enzymic oxidation of the other compounds.

Sulphydryl compounds as substrates

A crystalline sample of glutathione prepared by Pirie's method [1930: 1932] was employed and a commercial sample of cysteine hydrochloride was found to yield satisfactory results. Preliminary experiments have shown that at pH 6·0 oxidation was hardly, if at all, accelerated by the presence of the juice at the concentrations used (10 units of enzyme and 0·01 N substrate). Further trials have, however, shown that at pH 7·4 the oxidation was markedly catalysed and consequently the experiments were carried out at this pH. As no quantitative
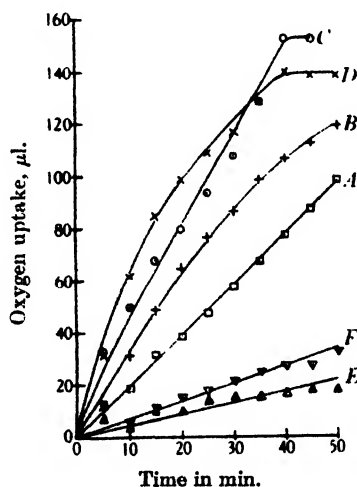
Fig. 15. Oxidation of 0·01 N solutions of glutathione and cysteine.

(a) In the presence of dialysed cucumber juice $\quad\begin{cases} \square - \square \ (A) \ \text{GSH.} \\ + - + \ (B) \ \text{cysteine.} \end{cases}$

(b) In the presence of dialysed cucumber juice with the addition of 10 mg. ascorbic acid per 100 ml. $\quad\begin{cases} \odot - \odot \ (C) \ \text{GSH.} \\ \times - \times \ (D) \ \text{cysteine.} \end{cases}$

(c) In the absence of enzyme (controls) $\quad\begin{cases} \triangle - \triangle \ (E) \ \text{GSH} \\ \triangledown - \triangledown \ (F) \ \text{cysteine.} \end{cases}$

assessment of glutathione can be obtained by titration with indophenol, determinations of the substrate concentrations were carried out iodimetrically. The various concentrations of cysteine hydrochloride were made up on the assumption that this compound was pure.

Owing to the fact that undialysed cucumber juice usually contains traces of dehydroascorbic acid, the possibility could not be excluded that the oxidation of the sulphydryl compounds did not take place directly by the enzyme, but was due to the action of dehydroascorbic acid [cf. Hopkins & Morgan, 1936].

Additional experiments were therefore performed with dialysed juice. It will be seen from Fig. 15 that although the addition of 10 mg. of ascorbic acid to 100 ml. of the dialysed juice approximately doubled its oxidizing activity, both sulphydryl compounds were oxidized even in the absence of ascorbic acid at pH 7·4; the dialysed juice contained less than 0·1 mg. of dehydroascorbic acid per 100 ml.

A point of interest emerged from these experiments, namely that when different samples of dialysed juice were employed the relative rates of oxidation of the sulphydryl compounds and of l-ascorbic acid varied (Figs. 16 and 17).
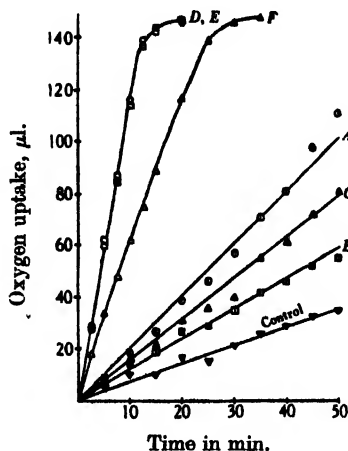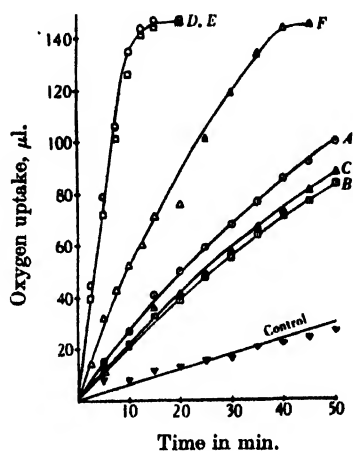
Fig. 16.　　　　　　　　　　　　Fig. 17.

Fig. 16. Oxidation of 0·01 N solutions of glutathione and l-ascorbic acid in the presence of equal amounts of different samples of dialysed cucumber juice.

	GSH	l-Ascorbic acid
Sample I	⊙—⊙ (A)	○—○ (D)
,, II	⊡—⊡ (B)	▢—▢ (E)
,, III	△—△ (C)	△—△ (F)

Fig. 17. Oxidation of 0·01 N solutions of cysteine and l-ascorbic acid in the presence of equal amounts of different samples of dialysed cucumber juice.

	Cysteine	l-Ascorbic acid
Sample I	⊙—⊙ (A)	○—○ (D)
,, II	⊡—⊡ (B)	▢—▢ (E)
,, III	△—△ (C)	△—△ (F)

This suggests that in all probability different enzymes in the juice were involved in the oxidation of glutathione and cysteine on the one hand, and of l-ascorbic acid on the other.

DISCUSSION

The information obtained in this investigation suggests that the action of the ascorbic acid oxidase is not confined to l-ascorbic acid (I) and d-gluco-ascorbic acid (II), cyclic dienols with an oxygen bridge. It was shown that the oxidations of a cyclic dienol without an oxygen bridge, reductic acid (III) and an acyclic dienol, reductone (IV),· both of which were capable of reducing indophenol, were catalysed by the enzyme. In most of the compounds the rate of oxygen absorption showed an inhibition as the oxidation proceeded. The oxidation of l-ascorbic acid and, as previously found [Johnson & Zilva, 1937, 2],

that of its analogues with the oxygen ring to the right of the carbon chain, were, however, directly proportional to the time.
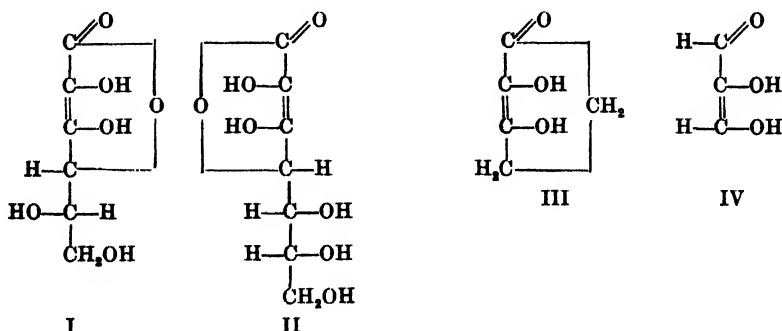
Amongst all the other substrates used in this investigation reductone stood out in its behaviour towards increasing enzyme concentrations in so far that beyond a certain stage the addition of more enzyme brought about very little response in the O_2 uptake (Figs. 9 and 11). The effect was specially noticeable when undialysed juice which showed greater activity towards reductone was employed. A possible explanation of this phenomenon may lie in the fact that reductone can exist in at least two tautomeric forms IV and VI. The high reducing properties of the compound in solution are presumably due to IV which would form the effective substrate and the concentration of which would therefore be controlled by the velocity of the tautomeric conversion. At high enzyme concentrations this may become the limiting factor in the rate of oxidation. The behaviour of reductone towards ascorbic acid oxidase is similar to that of fructose fermented by increasing quantities of yeast as observed by Hopkins & Roberts [1935, 1, 2]. In this case the formation of the fermentable component in the fructose solution could actually be observed by the rate of mutarotation.

In the compounds containing the 5-membered ring the O_2 uptake was consistent with the values calculated from the disappearance of the substrate on the assumption that two atoms of hydrogen were eliminated from each mol. In the case of reductone, however, the O_2 uptake was less than that calculated. This suggests that some of the substrate was utilized in a side reaction. By analogy with the easy conversion of methylglyoxal into lactic acid, the transformation of VI into glyceric acid (VII) provides a mechanism which would be consistent with such an assumption.
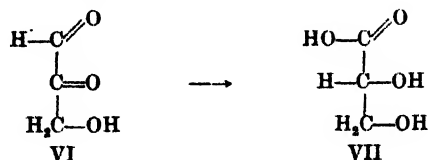
The oxidation of dihydroxymaleic acid by the cucumber juice, unlike those of reductone and reductic acid, is not due to the presence of ascorbic acid oxidase but to another enzyme system, possibly a phenolase. The high O_2 uptake and the production of CO_2 in this case suggest a breakdown either of the acid or of a primary oxidation product, with the formation of a substance capable of absorbing O_2. It is well known that the products of oxidation of dihydroxymaleic acid depend on the nature of the oxidizing agent. Thus the halogens

oxidize it (V) to dihydroxytartaric acid (VIII) which easily decomposes into hydroxymalonic acid (IX) and CO_2, whilst ferric salts oxidize it to glyoxalcarboxylic acid (X), CO_2 and water. This behaviour of dihydroxymaleic acid towards oxidizing agents provides some suggestion for its more complete degradation in the presence of the juice.

$$
\begin{array}{cccc}
\text{HO—C—COOH} & & \text{(HO)}_2\text{—C—COOH} & & \text{COOH} \\
\| & \rightarrow & | & \rightarrow & | & + \ CO_2 \ + \ H_2O \\
\text{HO—C—COOH} & & \text{(HO)}_2\text{—C—COOH} & & \text{HO—C—HCOOH} \\
\text{V} & & \text{VIII} & & \text{IX}
\end{array}
$$

$$
\begin{array}{ccc}
\text{HO—C—COOH} & & \text{O=C—COOH} \\
\| & \rightarrow & | & + \ H_2O \ + \ CO_2 \\
\text{HO—C—COOH} & & \text{H—C=O} \\
\text{V} & & \text{X}
\end{array}
$$
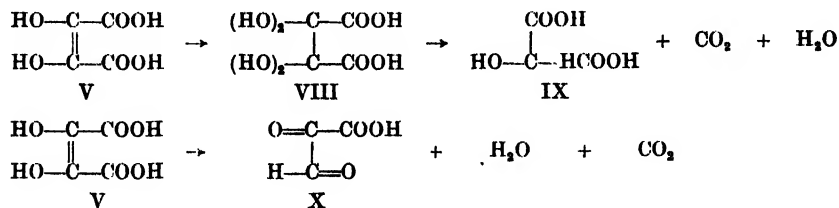
It is of interest to note that the enzymic oxidation of *l*-ascorbic acid *in vivo* has so far been indicated to be due only to the indirect action of a phenolase [Zilva *et al.* 1938] and not to the direct oxidation by the ascorbic acid oxidase. This does not, however, exclude the possibility that the oxidase functions also *in vivo*. The marked capacity of the enzyme to act in dilute solutions of *l*-ascorbic acid as compared with other substrates in fact strongly favours this view, since the vitamin is present in plants in very low concentrations. Even in some of the exceptionally rich sources such as the juice of the mango (Alphonso variety) it is present only to the extent of $0.01 \ N$. It would indeed be surprising if the ascorbic acid oxidase were not involved in any of the metabolic functions of the plant.

SUMMARY

Dialysed cucumber juice, apart from its ability to oxidise the ascorbic acid analogues directly, also oxidizes reductone and reductic acid.

Whilst in the compounds containing the five-membered ring the O_2 uptake agrees with the values calculated from the disappearance of the substrate, in the case of reductone the O_2 uptake is less than that calculated. It is suggested that some of the substrate is utilized in a side reaction.

Low concentrations of *l*-ascorbic acid are much more readily oxidized by dialysed juice than *d*-gluco-ascorbic acid, reductic acid and reductone, indicating a greater affinity of the enzyme for the naturally occurring vitamin.

Undialysed, but not dialysed, juice oxidizes dihydroxymaleic acid. Dialysed juice, however, regains its oxidizing activity towards this substrate on addition of catechol or phenol.

In the oxidation of dihydroxymaleic acid there is a formation of CO_2 and a higher O_2 uptake than that calculated from the disappearance of the substrate. The possible mechanism involved in this more complete degradation of the substrate is discussed.

Dialysed cucumber juice is capable of oxidizing glutathione and cysteine. The enzyme responsible for this oxidation appears not to be identical with the ascorbic acid oxidase present in the juice, since the relative rates of oxidation of the sulphydryl compounds and of ascorbic acid vary with different samples of dialysed juice.

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CCXLIX. UTILIZATION OF POLY-GLYCEROL ESTERS

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THE polymerization of glycerol to di- and tri-glycerol and to more highly polymerized glycerols has been described by a number of investigators, including Harris [1935], who has also prepared mixed esters of these polyglycerols with fatty acids. The large variety of esters which may be thus synthesized, though in many respects resembling fats, are distinguished by their wetting, emulsifying and other capillary-active properties, these being apparently related to the structure of the molecule in that it has a lipophilic group, in the form of a long-chain fatty acid residue, and hydrophilic groups in the form of free hydroxyl at or near an end of the molecule. These surface-active functions of the poly-glyceride esters are being utilized in various industries, and Harris [1933] describes their mechanism and suggests the use of these esters as emulsifying agents in edible emulsions. He also suggests their use in small percentages in margarine with the object of reducing the spattering of the margarine during frying. Because of the uniqueness of this group of compounds and their utility, it seemed of interest to investigate their utilization in the animal organism, the present report being principally concerned with growth and faecal excretion of fat.

EXPERIMENTAL

Albino rats were used, the stock in this laboratory being a pure inbred Wistar strain, reared under favourable conditions. The basic diet and the one fed to the controls consisted of ground Purina chow, to which was added a dried milk preparation (5 %), yeast (1 %) and water to proper consistency. For the experimental animals the basic ration was supplemented either with lard (5 or 10 %), or polyglycerol ester (5 or 10 %). In one large series of experiments the diet was begun soon after weaning (25–30 days old) and continued for as long as 14 months. Observations on over 100 rats revealed that both in the lard and polyglycerol ester-fed rats the rate of growth was normal or better than normal, the criterion for normal growth being that of the controls, which in our experience exceeded somewhat the standards obtained for the Wistar colony, as recorded by Greenman & Duhring [1931].

Generally the lard-fed rats attained somewhat greater weights than the rats in the polyglycerol ester group, the difference being apparently related to the much greater accumulation of fat in the depots (perirenal, mesenteric, omental, genital etc.) in the former. Histological examination of the tissues disclosed no abnormalities in either of the experimental groups. Rats were maintained successfully on the polyglycerol ester and lard diets through three generations,

the diets being continued without interruption throughout, including the periods of gestation and lactation. A number of typical growth curves of first generation rats fed with polyglycerol ester and lard are given in the accompanying charts.
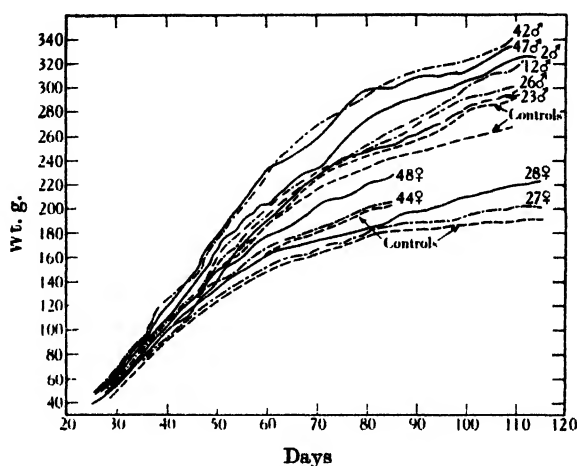
Fig. 1. Growth curves of rats: solid lines, 10% lard; dots and dashes, 10% polyglycerol ester; broken lines, controls.
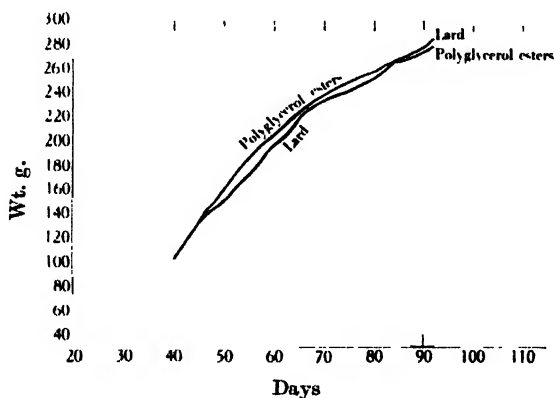
Fig. 2. Composite growth curves of three rats fed polyglycerol ester and three which received a comparable amount of lard (10% of diet). The six rats were male litter-mates and the offspring of parents reared on a polyglycerol ester-containing diet.

The fatty acids of the polyglycerol preparation used in these studies were chiefly stearic and oleic. The compound had i.v. 31·6 and sap. equiv. 143. Its utilization was determined in a series of mature male and female rats as indicated in Table I. During the experimental period of 24 days a record was kept of the daily food consumption. The faeces for each 3-day interval were collected separately and weighed, the excreta for eight such periods being combined and sampled for analysis. In the control group the data are based on 21 days of observation, as one 3-day specimen was discarded owing to incomplete collection. There were no striking differences in the water contents of the faeces of the various groups. It is to be noted further that the ratio of food consumed to the weight of faeces varied within narrow limits and was not significantly

Table I. *Utilization of fat in rats receiving supplements of lard or polyglycerol ester*

Exp.	Dura-tion days	Rats no. and sex	Initial and final wt. g.	Food con-sumed g.	Faeces g.	Faeces % of food consumed	Faecal fat %	Faecal fat I.V.	Depot fat I.V.
Lard, 5%	24	45 ♂ 46 ♂	369–390 410–448	1398	224	16·0	3·96	76·4	76·6 75·5
		64 ♀ 65 ♀	215–240 209–232	961	172	17·9	2·29	89·5	77·3
Lard, 10%	24	25 ♂ 26 ♂	333–340 330–338	1018	178	17·5	3·82	78·7	—
		3 ♀ 28 ♀	225–245 227–208	812	127	15·6	2·30	87·2	78
Polyglycerol ester, 5%	24	62 ♂ 63 ♂	273–322 290–339	1164	194	16·6	4·40	66·6	—
		35 ♀ 5 ♀	222–225 253–256	857	157	18·3	2·63	81·2	78·1
Polyglycerol ester, 10%	24	21 ♂ 23 ♂	356–360 309–348	1047	166	15·8	6·26	58·3	68·8
		13 ♀ 14 ♀	193–210 172–200	704	111	15·8	3·41	63·8	76·6 78·4
Control	21	51 ♂ 52 ♂	332–358 322–332	925	126	13·5	1·66	77·3	—
		39 ♀ 32 ♀	216–227 213–225	865	145	16·8	2·24	91·2	67·3

different in the lard and polyglycerol ester groups. In the rats receiving polyglycerol ester, the amounts of faecal lipins were somewhat greater than those in the lard group, which in turn exceeded the values found in the controls. However, even if all the ether-soluble material of the faeces in rats 21, 23, 13 and 14 represented unabsorbed polyglycerol compound, the results would nevertheless indicate 90% utilization in the males and 94·62% in the females. But judging from the faecal fat in the controls as well as the I.V., it is conservative to estimate that at least 95–98% of the polyglycerol esters of the rations were digested and absorbed even when they constituted 10% of the food mixture.

Polyglycerol esters of oleic and linoleic acids. The observation by Hansen [1933] that in infantile eczema the serum fatty acids have an abnormally low I.V. and his subsequent statement [1933–4] concerning the therapeutic action of unsaturated oils, such as linseed oil, stimulated in us further interest in the problem. It seemed that if unsaturated fatty acids actually exercised a beneficial effect their administration in polyglycerol combination would be of advantage, if for no other reason than the ease of preparing suitable emulsions. Accordingly, polyglycerol was esterified with commercial oleic acid and with a good grade of linseed oil. The constants for oleic acid ester were: I.V. 69·4, sap. equiv. 151. Those for the linseed oil fatty acid ester mixture were 116·7 and 137·2, respectively. The esters were fed at levels of 5% as indicated in Table II. The controls were maintained on the basal ration, while for further comparison other groups of rats received butter, 10%; oleomargarine, 10%; linseed oil, 5%; cocoa butter, 5%. As we were especially interested in the utilization of linseed oil and the polyglycerol esters of the unsaturated fatty acids in the young, the rats selected for this work were not more than 40 days old (oleic and linoleic ester groups and the controls, Table II, were 30 days old at the beginning of the experiment). It will be noted that the rate of growth was normal, or somewhat

Table II. *Utilization of various fats, including polyglycerol esters of oleic and linoleic acids*

Exp.	Dura-tion days	Rats no. and sex	Initial and final wt. g.	Food con-sumed g.	Faeces g.	Faeces % of food consumed	Faecal fat %	Faecal fat i.v.	Depot fat i.v.	Depot fat Sap. equiv.
Oleic ester, 5%	24	68 ♂ 69 ♂	56–160 54–158	971	157	16·2	4·45	86·5	73·5 80·5	216·5 205·5
		70 ♀ 71 ♀	53–142 50–144	783	116	14·8	4·9	92·3	76·9	—
Linoleic ester, 5%	24	72 ♂ 73 ♂	58–162 56–152	932	124	13·3	3·79	77·6	81·2	198·5 197·5
		74 ♀ 75 ♀	45–128 50–126	737	132	17·9	4·34	87·2	91·5 81·1	—
Control	24	76 ♂ 77 ♂	52–159 55–160	945	160	17·05	4·14	80·5	73·2	199·5
		78 ♀ 79 ♀	56–137 58–148	927	191	20·6	2·83	89·7	81·2	202
Butter, 10%	21	80 ♂ 81 ♂	67–151 75–165	878	125	14·3	4·16	57·0	76·5	201
		82 ♀ 83 ♀	64–140 63–124	827	138	16·7	3·58	72·5	65·1	199
Oleomargarine, 10%	21	86 ♀ 87 ♀	53–128 60–143	710	127	17·6	2·94	73·0	79·6	198·5
		89 ♂ 90 ♂	76–163 78–174	874	137	15·7	3·59	72·1	94·0	192·5
Linseed oil, 5%	24	91 ♀ 92 ♀	73–132 75–140	545	107	19·6	4·18	78·2	81·5	182·5
		93 ♂ 94 ♂	70–150 78–180	944	152	16·1	5·11	65·1	82·8	—
Cocoa butter, 5%	24	95 ♀ 96 ♀	64–118 74–149	748	117	15·7	4·54	67·0	79·9	203·5

better than normal in all groups (note especially the excellent growth of the polyglycerol ester groups). As shown by the data for faecal lipins, there was almost complete utilization of the polyglycerol esters of oleic and linoleic acids, as well as of the linseed oil and other fats used. Under the conditions of the experiments, the composition of the depot fat was only slightly influenced by the kind of fat incorporated in the diet.

The faeces were analysed essentially according to the method of Sperry & Bloor [1924], while for the analysis of the tissues the methods of Reed and associates [1930; 1932] were used.

COMMENT

The composition of the "fat" of the faeces, while not altogether unrelated to that of the fat consumed, is remarkably constant and independent of the diet, as has been shown by a number of investigators [Hill & Bloor, 1922; Holmes & Kerr, 1923–4; Sperry & Bloor, 1924]. Only under conditions in which digestion and absorption of fat are diminished, whether through disease or otherwise, does the composition of the faecal fat reflect that of the diet. Accordingly, analysis of the faeces may afford an accurate measure of the utilization of a fat in the alimentary tract. In the light of existing knowledge the results of the present study demonstrate that the coefficients of digestibility (including absorption) of the polyglycerol esters of stearic, oleic and linoleic

acids are essentially the same as those of naturally occurring fats (compare with data of Holmes & Kerr [1923–4]. *In vitro* experiments have confirmed the digestibility of these esters by lipase.

SUMMARY

Compounds produced by partial esterification of the polymerides of glycerol (diglycerol, triglycerol, tetraglycerol etc.) with fat-forming fatty acids, when fed at levels of 5 and 10% of the diet were found to be as well utilized in the alimentary tract of the albino rat as were naturally occurring fats. No untoward effects, as regards growth, reproduction, lactation and the microscopic appearance of the tissues, were observed as the result of long-continued feeding of these compounds.

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CCL. A NOTE ON THE DEXTRAN PRODUCED FROM SUCROSE BY *BETACOCCUS ARABINO-SACEOUS HAEMOLYTICUS*

BY MAURICE STACEY AND FREDERICK ROBERT YOUD

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DURING the large scale production of the dextran from sucrose by *Betacoccus arabinosaceous haemolyticus* (*Leuconostoc dextranicum*) using the method described previously by Carruthers & Cooper [1936], unforeseen and inexplicable irregularities of growth occurred, so that a further investigation of the metabolic requirements of the organism was deemed necessary. It has been possible to develop a more satisfactory medium for the dextran production. In this medium use is made of commercial maple syrup to provide a reliable supply of the "accessory growth substance" (which may be simply a source of available nitrogen) together with an increase to 20% in the concentration of sucrose.

Sufficiently large quantities of the dextran to facilitate its chemical examination were isolated and it was purified without the use of heat, acid or alkali. Previous difficulties in removing protein impurities were minimized by a preliminary filtration of the dextran solution through a kieselguhr pad. In purified samples, despite the fact that the usual protein tests were negative, a small fragment of nitrogenous material (N, 0·5% approx.) persisted. The dextran could be obtained N-free only after mild acid or alkaline treatment. Complete elimination of the nitrogenous constituent was accompanied by a decrease in viscosity of the dextran. This phenomenon was especially marked when a solution was treated for a short period at 100° with weak acid. In such a case the nitrogenous constituent separated from the clear solution in the form of a flocculent precipitate—a phenomenon which we have frequently observed with other polysaccharides of microbiological origin, notably with Types I and II pneumococcus specific polysaccharides.

In the case of the dextran produced by *Leuconostoc mesenteroides*, Fitzgerald [1933] found that its immunizing properties, originally investigated by Zozaya [1932, 1], were due entirely to a nitrogenous constituent since all antigenic activity disappeared when the N content of the dextran fell below ·0·2%. In a similar manner certain antigenic properties ascribed to gum arabic have also been shown to be connected with small amounts of a nitrogenous constituent [Uhlenhuth & Remy, 1935]. The significant decrease in viscosity of the dextran after removal of its nitrogenous fraction may indeed indicate that the latter, although comparatively small, is an actual constituent of the dextran macromolecule. The possible significance of such a compound acting as a stabilizer of the macromolecular state of the polysaccharide, and thereby conserving certain of its antigenic properties, may be worthy of investigation. In this connexion it is of interest to recall the experiments of Zozaya [1932, 2] who claimed to have endowed otherwise inactive polysaccharides with antigenic activity by injecting them as a suspension in collodion.

The relationship between the acetyl content of dextrans and their antigenic properties is under investigation in another Laboratory [Fowler *et al.* 1937],

so that it is of interest to report that the dextran described in this paper and isolated under the least drastic conditions contained no acetyl residues.

A preliminary account of the structure of the dextran has recently been published from these laboratories [Peat *et al.* 1938]. It has been established that this polysaccharide is constituted of a linear chain of several hundred glucose units mutually linked through the 1:6-positions. This is the gentiobiose linkage Further details of the structural investigations will be published later.

EXPERIMENTAL

Following the method of Carruthers & Cooper [1936] for dextran production it was observed that in some flasks growth and viscosity were readily produced whilst in others, prepared in an apparently identical manner, little or no growth took place. The irregularity became particularly marked when the volume of metabolism solution was increased beyond 100 ml. and also after repeated subculturing of the organism. In an investigation into the cause of this phenomenon the following points emerged.

(1) The *p*H values of the medium were identical in both viscous and weak cultures during and after growth, indicating that inhibition of dextran formation was not due to acid production.

(2) Sterilization of sucrose and peptone solutions separately, followed by aseptic mixing before inoculation, gave increased yields of dextran but growth was still irregular.

(3) Malt extract, lucerne root extract and yeast-water failed to provide a growth stimulant.

(4) Molasses extract and Demarara sugar gave inconsistent results and, moreover, when the dextran was formed it was heavily pigmented and difficult to purify.

(5) A medium containing 20% sucrose and 5% commercial maple syrup gave satisfactory results. The dextran formed in this case was free from pigmented material and was readily purified.

Large-scale dextran production

Peptone solution (double strength) and sucrose solution (40%) containing maple syrup (5%), were steamed separately on two successive days and then mixed aseptically in amounts of 100 ml. of each in 500 ml. conical flasks. After a third steaming the flasks were cooled and inoculated heavily, using sterile pipettes, with a viscous 48-hr.-old culture of *L. dextranicum*. After incubation at 30° for 10 days the viscous opalescent solution was filtered through cotton wool. The dextran was precipitated in the form of a white powder by the addition of 2 vol. of ethyl alcohol. It was filtered, washed with alcohol and dried *in vacuo*.

The crude material thus isolated amounted to 25% of the sucrose employed. It contained approx. 10% of bacterial protein the greater part of which was removed by repeated filtration of a dilute aqueous solution through kieselguhr. Final traces of protein material were removed by alcoholic fractionation, which, moreover, revealed that the dextran was essentially homogeneous. The purified dextran had the following properties: $[\alpha]_D^{20°}$ +180° in water (c, 1·0); ash, 0·4%; N, 0·25%; moisture, 8%. The small amount of nitrogenous constituent could not be removed by fractionation and its presence was characterized by a faint opalescence of an aqueous solution of the dextran. On heating such a solution with $N/10$ acetic acid or $N/10$ HCl a small amount of flocculent precipitate

separated and the opalescence disappeared. This treatment, as shown below, caused a considerable decrease in viscosity of the dextran:

	$[\alpha]_D$	Rel. vis. in water (1 % sol.)	Rel. vis. in saline (1 %) (1 % sol.)	N %	Reduction to Fehling's solution
Normal dextran	+180°	3·20	4·40	0·25	None
Dextran heated with $N/10$ HCl at 100° for 15 min.	+180°	1·01	1·15	0·0	None

Hydrolysis of the dextran was carried out using N H_2SO_4 at 100° for 18 hr., 5·0 g. yielding 5·6 g. of crystalline d-glucose. No other sugar was isolated.

SUMMARY

A reliable method is described for the large-scale production of dextran by *Leuconostoc dextranicum* and the purification and properties of the dextran are recorded.

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CCLI. INVESTIGATION OF A POLYSACCHARIDE PRODUCED FROM SUCROSE BY *BETA-BACTERIUM VERMIFORMÉ* (WARD-MEYER)

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WE have been able to examine a polysaccharide formed from sucrose by an organism (*Betabacterium vermiformé*) recently studied by Prof. A. J. Kluyver, to whom we are greatly indebted for a culture. The organism was isolated by Mr H. D. Meyer (private communication) in the course of an extensive and careful bacteriological examination of "Tibi-complex". The latter originates in Mexico where it occurs in the form of hard granules on the disk-shaped leaves of the *Opundia* plant. It has long been used by the natives for the preparation of a beverage by fermentation and is analogous to the classic example, the Kephir yeast of the Arabs. As in the case of Kephir, Tibi fermentation is due to the symbiotic action of a yeast and a bacterium. The bacterium isolated by Meyer appeared to be identical with that isolated by Ward [1892] from the Ginger-beer plant.

On a sucrose-gelatin medium, the bacterium readily formed colonies surrounded by a highly viscous secretion and its growth on gelatin slopes closely resembled that of *Betacoccus arabinosaceous anhaemolyticus* (*Leuconostoc mesenterioides*). Meyer was able to show that the viscous secretion consisted mainly of a polyglucose with properties strikingly similar to those of the dextran synthesized by *B. arab. anhaemolyticus* [Tarr & Hibbert, 1931]. We have been able to confirm the finding that it is indeed a dextran, resembling closely in physical properties the dextrans from both *B. arab. haemolyticus* and *B. arab. anhaemolyticus*.

Our preliminary investigations were concerned with production of the polysaccharide in a liquid medium. The medium adopted for preparing the dextran from *B. arab. haemolyticus* [Stacey & Youd, 1938] was unsuitable for the growth of *Betabact. vermiformé* although when a satisfactory substrate for the latter was devised certain similarities in the growth of the two organisms were observed.

Both dextrans could be synthesized from sucrose only; moreover, in each case it was mainly the glucose constituent of the sucrose molecule which appeared to be utilized in the growth. For consistent dextran production both organisms required a source of available nitrogen which was best supplied by maple syrup to *B. arab. haemolyticus* and by yeast extract to *Betabact. vermiformé*. The polysaccharide could conveniently be prepared in yields amounting to 20% of the sucrose employed in a simple yeast extract-sucrose medium. The growth reached a maximum in about 10 days and was quite uniform even in large volumes of liquid—a property which facilitated the large-scale dextran production.

Separation of the polysaccharide was effected by alcoholic precipitation followed by washing and drying by the usual methods. The crude material contained approx. 0·2% N and was insoluble in water. This insolubility differentiates it from the *Leuconostoc* dextrans which are readily soluble in water.

During growth a large proportion of the dextran became insoluble in the metabolism solution and after isolation and drying it could not be dissolved even in boiling water. On dissolving this insoluble material in strong acid or alkali about 5% of bacterial "debris" separated and was removed in a centrifuge. Thereafter the dextran, precipitated by alcohol, was now water-soluble, N-free, showed $[\alpha]_D + 180°$ in water and was indistinguishable in physical properties from the *Leuconostoc* dextrans.

From the products of acid hydrolysis, crystalline glucose (in 93% yield) was the only sugar isolated. Structural investigation has revealed that the main part of the molecule is constituted of α-glucose units mutually linked through the 1:6-positions, i.e. the linkage which obtains in gentiobiose [cf. Peat *et al.* 1938]. These and other investigations will be described later.

EXPERIMENTAL

Stock cultures of *Betabacterium vermiformé* received from Prof. A. J. Kluyver were maintained on gelatin slopes of the following composition: KCl, 0·5%; Na_2HPO_4, 0·2%; sucrose, 10%; gelatin, 15%; yeast-water, 10%.

In a search for optimum conditions for dextran production in liquid media, the following main facts were established.

(1) The organism could synthesize the dextran from sucrose only, the yield increasing to a maximum at a sucrose concentration of 20%. Complete inhibition of growth occurred when the sucrose was replaced by glucose, fructose, maltose, lactose, glycerol, mannitol, sorbitol, inulin or laevan.

(2) No dextran formation took place in a sucrose-peptone medium in the absence of other sources of N the best of which was 10% yeast-water extract. Addition of maple syrup or beet sugar molasses gave unsatisfactory dextran production in the absence of an extra N supply, but in the presence of peptone (0·1%) or asparagine (0·1%) production was good.

(3) Tomato extract and a crude vitamin B concentrate were able also to provide the source of available N.

(4) The yield of dextran from a 10% yeast-water-sucrose medium was slightly increased by the presence of urea (0·1%) or succinamide (0·1%) or creatinine (0·1%). Glycine, *l*-leucine, malonamide and *d*-alanine appeared to act as growth inhibitors.

(5) During growth the pH of the metabolism solution was unchanged so that addition of $CaCO_3$ offered no advantage.

(6) No dextran was produced when the organism was grown in the medium which had proved satisfactory for the production of dextran from *Leuconostoc dextranicum*.

(7) The volume of metabolism solution did not materially affect the rate or amount of dextran formed in a suitable medium.

Large-scale production

Satisfactory results were obtained with the following medium: yeast-water, 10%; KCl, 0·5%; Na_2HPO_4, 0·2%; sucrose, 20%. The medium was distributed in 600 ml. quantities in 1-litre conical flasks and steamed on three successive days. The flasks were inoculated, using small sterile pipettes, from a viscous 48 hr.-old culture of *Betabact. vermiformé* growing in test tubes in an identical medium. After incubation at 30° for several days the medium became very viscous and a dense gummy layer was formed at the bottom of each flask. (In preliminary experiments this material was isolated separately by decantation and was shown to consist of crude dextran.) After 10 days' incubation the

contents of the flasks were well shaken and run through cotton wool into 2 vol. of alcohol.

The crude dextran which was thrown down was washed repeatedly with alcohol, ground to a fine powder in a mortar and dried in a vacuum. The average yield amounted to 20% of the sucrose used. A considerable amount of fructose was isolated from the mother liquors [cf. Carruthers & Cooper, 1936] indicating that the dextran was probably synthesized from the glucose moiety of the sucrose molecule.

Purification and properties

The crude dextran (N 0·2%) was completely insoluble in water and did not swell in boiling water. It dissolved readily in $5N$ NaOH and in $10N$ HCl and showed optical properties as follows: $[\alpha]_D^{20°}+177°$ in $10N$ HCl (c, 0·68); $[\alpha]_D+179°$ in $5N$ NaOH (c, 0·90). It was rendered soluble in water by the following method suggested by Dr F. Smith: dextran (3 g.) was stirred vigorously in cold conc. HCl (20 ml.) and when evenly suspended was centrifuged. A small amount of amorphous material was thrown down and the clear supernatant liquid was poured into excess of ethyl alcohol. The dextran was precipitated in the form of a fine white powder which was washed free from acid by alcohol and ether and dried in a vacuum. Yield, 2·8 g.

The product was now readily soluble in water. It had no reducing action on Fehling's solution, contained no N and its specific rotation was unaltered by the acid treatment. $[\alpha]_D^{20°}+175°$ in $10N$ HCl (c, 1·0); $[\alpha]_D^{20°}+177°$ in water (c, 1·3); $[\alpha]_D+179°$ in $5N$ NaOH (c, 0·95).

The insoluble gummy layer usually found at the bottom of the metabolism solution and mentioned previously, was treated with conc. HCl in the manner described and yielded a product, $[\alpha]_D^{20°}+179°$ in $5N$ NaOH (c, 1·3), identical with that in the metabolism solution. An average sample of dextran had the following properties: $[\alpha]_D^{20°}+180°$ (c, 1·3); ash, 0·23%; I.V. 4·7. On hydrolysis with N H_2SO_4 at 100° the following polarimetric changes were observed: $[\alpha]_D^{20°}+183°$ (0 min.), $+148°$ (45 min.), $+142°$ (80 min.), $+132°$ (115 min.), $+117°$ (190 min.), $+98°$ (310 min.), $+85°$ (370 min.), $+79°$ (430 min.), $+66°$ (490 min.), $+54°$ (925 min., equilibrium value).

From the hydrolysate crystalline d-glucose, $[\alpha]_D+52·4°$, was isolated in 93% yield. The chemical investigation of the dextran will be reported at a later date.

SUMMARY

A practicable method is described for the preparation of a new polysaccharide from sucrose by means of an organism *Betabacterium vermiformé* (Ward-Meyer). The polysaccharide shows a close resemblance in properties to the dextrans synthesized by the *Leuconostoc* species.

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CCLII. POLYLAEVANS FORMED BY THE CARBOHYDRATE METABOLISM OF CERTAIN BACTERIA

By STIG VEIBEL

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ØRSKOV [1930; 1931; 1936; 1938; Ørskov & Poulsen, 1931] has stated that when certain Gram-negative organisms found in milk and certain soil bacteria (*Actinomycetes*) are grown on sucrose- or raffinose-containing subtrates, polysaccharides are formed abundantly. The substances formed have, however, not been examined chemically, and their nature as polysaccharides has been deduced from their appearance alone. It was therefore thought to be of interest to examine them more thoroughly, and the investigation here reported shows that the three micro-organisms hitherto studied do in fact form polylaevans and most probably the same laevan in each case.

(1) *Substance from milk bacteria* [*Ørskov, 1931*]

The micro-organisms were grown on 2% sucrose-agar. After 2 days the bacteria were separated by filtration and the "colonies" of polysaccharide remaining in the somewhat opalescent liquid were precipitated by addition of alcohol and ether. The supernatant liquid was decanted and the precipitate was washed with physiological salt solution, separated by centrifuging and dried to constant weight in a vacuum desiccator over conc. H_2SO_4.

Analysis showed that the substance isolated had the composition of a polysaccharide, contaminated with a small amount of protein possibly originating from residual bacteria or from cellular nuclei in the "colonies" of polysaccharide. (Found: C, 44·32; H, 6·41; N (Kjeldahl), 0·59%. $(C_6H_{10}O_5)_n$ requires C, 44·42; H, 6·23%.) The polysaccharide is practically insoluble in all solvents. It does not reduce Fehling's solution, but after hydrolysis with boiling dilute acid it gives strongly reducing products.

0·54 g. of the polysaccharide was heated for 3 hr. on the steam bath with 40 ml. water and 5 ml. N HCl. The solution was left at room temperature overnight; next day it was made up to 50 ml. in a measuring flask, filtered from a trace of flocculent precipitate and its optical rotation determined; $\alpha_D^{20°}$ was $-1·77°$ (c (calc. as monosaccharide) $=1·2$, $l=2$), whence $[\alpha]_D^{20°} = -74°$. This is a minimum value and it is some 20% lower than the specific rotation of equilibrium fructose, but as already pointed out the polysaccharide was not N-free; it may therefore be assumed with some probability that the polysaccharide is a (slightly impure) fructose anhydride (laevan).

In order to confirm this supposition, a solution of 1·5 g. polysaccharide was neutralized with NaOH, concentrated in a vacuum at 35–40° to low volume and finally dried in a vacuum desiccator over H_2SO_4. The residue, 1·2 g., was treated with dry acetone and conc. H_2SO_4 as recommended by Ohle & Koller [1924] for the preparation of β-diacetonefructose. 0·9 g. of a compound with M.P. 95–96° and $[\alpha]_D^{20°} = -38·6°$ (alcohol, $c=1·166$) or $-34·3°$ (water, $c=1·808$) was obtained. Ohle & Koller [1924] obtained from 10 g. of fructose 6·5 g. of diacetonefructose

with M.P. 97° and $[\alpha]_D^{20°} = -36.69°$ (alcohol, $c=1.172$) or $-26.17°$ (water, $c=1.856$); they indicate, however, that the specific rotation in aqueous solution depends on the concentration; for $c=3.161$ they found $[\alpha]_D^{20°} = -32.9°$.

For comparison we prepared β-diacetonefructose from authentic fructose. From 2.5 g. we obtained 1.4 g. of a preparation with M.P. 96-97°, i.e. nearly the same yield as that quoted by Ohle & Koller. The specific rotation of this preparation in aqueous solution did not depend on the concentration: for $c=3.193$ $[\alpha]_D^{20°}$ was $-34.0°$ and for $c=1.811$ $[\alpha]_D^{20°}$ was $-34.1°$, in agreement with Ault *et al.* [1935] who found $[\alpha]_D^{20°} = -34°$ (water, $c=0.9$). For an alcoholic solution with $c=1.183$ we found $[\alpha]_D^{20°} = -37.97°$ for the product from fructose. The values found by us for authentic diacetonefructose are thus identical with those found for the acetone derivative of the product of hydrolysis of the polysaccharide, which therefore may be assumed to be fructose.

Further confirmation of this assumption has been obtained by the method of Bridel [1930] for detection of glucose or galactose in plant materials, namely, solution of the substance in 70% methyl alcohol and examination of the influence of addition of emulsin (β-glucosidase). If a sugar, which is able to form a methyl glycoside under the influence of emulsin is present, an alteration of the optical rotation of the solution will take place.

The monosaccharide obtained from 4.65 g. of the polysaccharide was dissolved in 100 ml. 70% methyl alcohol and 0.2 g. of an emulsin preparation with sal. f. 0.091 was added. The solution was rotated mechanically for 14 days at 30°. Before addition of emulsin the solution had $\alpha_D - 5.17°$ and the rotation remained unaltered during the experiment. From this it may be concluded at least that glucose and galactose are absent from the solution, and the assumption that fructose is the sole product of hydrolysis gains in probability.

(2) *Substance from soil* Actinomycete, *strain 38.* [Ørskov, 1938]

The bacteria were grown as above. The polysaccharide formed in this case, although corpuscular, did not form "colonies"; it was isolated in the same manner as previously. 2 g. were hydrolysed by heating on the steam bath for 3 hr. with 150 ml. water and 5 ml. $N/10$ HCl. The solution was left overnight at room temperature and then neutralized with NaOH, filtered, evaporated in a vacuum at 40° to low volume and finally dried in a vacuum desiccator over H_2SO_4. The residue was a syrup, which was taken up in 30 ml. abs. alcohol; only a very little hygroscopic substance was left undissolved. The alcoholic solution was evaporated to dryness and 1.5 g. of non-hygroscopic substance were obtained. The M.P. was very unsharp, 80-95° (decomp.). Pure fructose melts at 102° or 95°. 0.0997 g. was dissolved to make 10 ml. aqueous solution. In a 2 dm. tube α_D was $-1.57°$, and next day had diminished to $-1.52°$, i.e. $[\alpha]_D^{20°} = -76.2°$. 1.5 g. of the substance yielded 1.05 g. of a diacetone derivative with M.P. 96-97° and $[\alpha]_D^{20°} = -38.76°$ (alcohol, $c=1.193$). This polysaccharide also seems therefore to be a laevan.

(3) *Substance from soil* Actinomycete, *strain 47.* [Ørskov, 1938]

The bacteria were grown and the substance isolated as in the case of strain 38. 2.0715 g. were heated for 1 hr. on the steam bath with 85 ml. water and 5 ml. N HCl. Next day the solution was filtered and made up to 100 ml. The rotation of the solution in a 2 dm. tube was $-3.24°$, whence $[\alpha]_D^{20°} = -70.4°$.

The solution was neutralized with NaOH and the monosaccharide was isolated as above. Yield *ca.* 2 g. Without purification the material was trans-

formed into a diacetone derivative, of which 1 g. was obtained with M.P. 95·5 — 96·5 and $[\alpha]_D^{20°} = -38·67$ (alcohol, $c = 1·170$). In this case too fructose seems to be the sole product of hydrolysis.

Comparison with other naturally occurring laevans. Acetylation and methylation

Polylaevans are rather commonly met with in nature. Inulin is the classical example, but in the last few years Schlubach and co-workers [1936; 1937, 1, 2, 3] have isolated a series of fructose anhydrides from different plants, and, which is particularly significant for us, Harrison *et al.* [1930] and Hibbert and co-workers [1930; 1931, 1, 2] have found polylaevans to be synthesized by various micro-organisms when they are grown in sucrose solutions.

The micro-organisms examined by Hibbert *et al.* were *Clostridium gelatige-nosum*, *Semiclostridium commune*, *Bacillus laevaniformans*, *B. hemiphloriae*, *Aspergillus sydowi*; the *subtilis* and *mesentericus* groups were specially prone to synthesize polylaevans and the most effective seems to have been *B. mesentericus* Trevisan which can bring about the synthesis from sucrose or raffinose but not from melezitose, lactose, maltose, xylose, glucose or fructose. Ørskov [1938] has found the same for the micro-organisms examined by him.

The polylaevans examined by Schlubach *et al.* were all soluble in water. Those isolated by Hibbert *et al.* were soluble in hot and slightly so in cold water. In most organic solvents they were insoluble, but they dissolved in hot glycerol or ethyleneglycol. These laevans, with acetic anhydride and pyridine, gave triacetates which, after treatment first with methyl sulphate and NaOH and finally with Purdie's reagent, gave trimethyl derivatives. The triacetates and trimethyl derivatives of the different laevans differed in solubility, specific rotation and M.P., the most important difference being that the triacetates of the plant laevans were laevorotatory, whilst those of the bacterial laevans isolated by Hibbert *et al.* were dextrorotatory.

We have therefore prepared the triacetates and trimethyl derivatives of our laevans and have compared their properties with those mentioned above.

(1) *Substance from milk bacteria*. The acetylation was carried out as indicated by Schlubach & Loop [1936]. A triacetate was formed which softened at 90–100° but was completely molten only at 160–170°. (Found: C, 49·73; H, 5·15; CH_3CO, 43·5 %. $(C_{12}H_{16}O_8)_n$ requires C, 50·00; H, 5·60; CH_3CO, 44·9 %.)

The triacetate was nearly insoluble in all solvents. Glacial acetic acid dissolved a small amount when warm, but most of it separated again on cooling; the cooled solution was dextrorotatory, having $\alpha_D + 0·06°$. In chloroform the product had $[\alpha]_D^{20°} + 11·2$, which is to be regarded with reserve as a minimum figure.

The methylation of the triacetate was carried out as indicated by Schlubach & Loop [1936]. The trimethyl derivative was dissolved in benzene and precipitated by light petroleum. A white, non-hygroscopic, amorphous powder was obtained, which softened at 122–125° and was completely molten at 140° $[\alpha]_D^{20°} = -60·9°$ ($CHCl_3$, $c = 2·086$). (Found: OCH_3 44·6 %. $(C_9H_{16}O_5)_n$ requires OCH_3 45·6 %.)

(2) *Substance from soil Actinomycete, strain 38*. The triacetate and trimethyl derivative were prepared as mentioned above. The trimethyl derivative softened at 122–125° and was completely molten at 138°. $[\alpha]_D^{21°} = -57·2°$ ($CHCl_3$, $c = 1·958$, $l = 2$). (Found: OCH_3, 46·3 %. Calc. OCH_3, 45·6 %.)

(3) *Substance from soil Actinomycete, strain 47*. The trimethyl derivative prepared as above had M.P. 138–140° (softening at 120–125°), $[\alpha]_D^{20°} = -58·8°$ ($CHCl_3$, $c = 1·360$, $l = 2$). On account of lack of material no methoxyl determination was made.

From the properties of the methyl derivatives it appears that all three preparations of polysaccharide are identical. In the table below we have collected the data for different laevans described in the literature.

| | M.P. | $[\alpha]_D$ | Triacetate | | Trimethyl derivatives | |
			M.P.	$[\alpha]_D$	M.P.	$[\alpha]_D$
Inulin [Hibbert et al., 1931]	—	−40° water	102–103°, soft 95°	−42·55°, CH₃COOH	138–140°	−50·2°, CHCl₃
Laevan [Hibbert et al., 1931]	—	−45·3° water	190°, soft 106°	+21°, CHCl₃	145–147°	−87 to −91°, C₂H₂Cl₄
Triticin [Schlubach & Peitzner, 1937]	—	−51·4° water	115° or 191°	−15·5°, CHCl₃	141–151°	−61·2°, CHCl₃
Sinistrin [Schlubach & Loop, 1936]	—	−44°	—	−23·5°, C₆H₆	—	−57°, CHCl₃
Asparagosin [Schlubach & Böe, 1937]	197–198° soft 170°	−32·4°	93°, soft 80°	−20·1°, CHCl₃	—	−47·8°, CHCl₃
Aspholedin [Schlubach & Lendzian, 1937]	—	−30·5°	—	−16·6°	—	—
Own preparations	—	—	160–170°, soft 90°	> +11°, CHCl₃	140°, soft 122°	−57 to −60°, CHCl₃

The dextrorotation of the triacetate points to the identity of our preparations with the laevans of Hibbert et al., whilst the rotations of the trimethyl derivates resemble more those of the plant laevans examined by Schlubach et al. Hibbert et al. states that their trimethyllaevans on hydrolysis yield 1:3:4-trimethyl-fructose, whereas trimethylinulin on hydrolysis yields 3:4:6-trimethylfructose. The amount of trimethyl derivative at our disposal did not permit an examination of the products of hydrolysis, but the investigation is being continued in this direction. We wish to point out, however, that the solubility of our preparations seems to differ both from that of plant laevans and from that of bacterial laevans hitherto described.

Thanks are due to Dr Ørskov, Statens Serum-Institut, Copenhagen, who called my attention to the problem and who has kindly taken the trouble of cultivating the micro-organisms necessary to produce the polysaccharide used in this investigation.

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CCLIII. A NEW METHOD FOR THE ISOLATION OF a- AND β-TOCOPHEROLS

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MOST attempts to isolate the antisterility factor from various oils have made use of saponification as the first stage, thereby effecting a concentration, at least theoretically, of about fourteen times in the case of wheat germ oil. In view of the unexpected sensitivity of the tocopherols to alkali [Moss *et al.* 1938], it seemed to us that a method avoiding the use of the latter would provide information as to the substances responsible for the biological activity of the natural oil, and might also give a better yield. Our experience has been that there are considerable losses during the saponification, even when the treatment is carried out with special precautions.

When it became apparent that the hydroxyl group of the tocopherol molecules was phenolic, it seemed reasonable to think that these compounds might be present in the original oil in the free state although it had usually been assumed, for no very clear reason, that they were esterified. Accordingly, the simple experiment was tried of adsorbing the tocopherols directly from a solution of the original oil. It was entirely successful.

Adsorption of wheat germ oil on Merck's alumina from solution in light petroleum has been found to effect a concentration of the active factor of about fourteen times, both biological and spectroscopic evidence indicating that the fraction recovered from the column contains the whole of the activity of the original oil. The experiments were performed on wheat germ oils from various sources and the results obtained have been uniform both qualitatively and quantitatively.[1]

The chromatogram shows three sets of bands. The first is a pinkish yellow system which moves rapidly down the column. The second is a yellow system which moves slowly downward and divides into upper and lower sections as more material becomes adsorbed; whilst the third is a deep orange yellow band which tends to stay near the top of the column. The tocopherols are associated with the second, slow-moving band system.

Since the great majority of the other constituents of wheat germ oil pass through the alumina column, it was found possible to use much larger amounts of oil for a given size of column than is the usual practice in the adsorption of unsaponifiable material. The maximum amount possible for a 45×4.5 cm. column was found to be 150 g. The limiting factor is the development of the chromatogram toward the bottom of the column, a process occurring more rapidly under the influence of a 10 % solution of oil in light petroleum than with the pure solvent. This circumstance is advantageous in that the usual prolonged washing with large volumes of solvent is unnecessary, the maximum possible

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[1] Since this paper was submitted for publication our attention has been drawn by Prof. A. R. Todd to a German patent by the I.G. Farbenindustrie taken out on 30 Jan. 1936 which covers a process of extraction very similar to our own.

separation between the bands occurring during the feeding of the column with the solution. It is then only necessary to wash the column with a small volume of solvent until the filtrate is colourless and practically free from fat, a column of the size mentioned above requiring 300–500 ml.

Eluted separately with mixtures of ether, benzene and methyl alcohol, containing about 80 % of ether, the two yellow bands of the slow-moving system yield yellow oils of which approximately 93 % appears to be triglyceride and esterified sterol. Examined spectroscopically in alcohol, the unsaponifiable materials of these fractions exhibit selective absorption in the ultra-violet, the upper one having a maximum at 295 mμ, and the lower at 292 mμ. Below the lower of the two bands there usually appears, during the washing of the column, a reddish-pink band with which is associated a substance having an ultra-violet absorption with a maximum at 288 mμ.

An attempt was then made to separate the tocopherol from the lower fraction by direct allophanation of the material eluted from the column. Spectroscopic examination before allophanation revealed selective absorption having a rather broad maximum between 286 and 289 mμ. It is reasonable to suppose that this maximum represents the absorption at 292 and 288 mμ shifted toward shorter wave-lengths by the presence of unsaponified fat.

The usual procedure was adopted in the allophanation and after removal of the cyamelide and benzene, three crystalline compounds were isolated from the oil by freezing the solution, first in acetone and then in light petroleum. All three substances were transparent to ultra-violet light and were not further examined. The uncrystallizable residues were adsorbed on alumina from light petroleum, yielding an upper fraction showing absorption with a maximum at 281–283 mμ; and a lower fraction having absorption with a maximum at 277–279 mμ. Readsorption of the two fractions produced no apparent alteration in the physical properties of the mixtures. It is known from the work of John [1937] and of Bergel, Todd & Work [1938] that allophanation causes a shift in the position of the maxima of ultra-violet absorption of 8–9 mμ toward shorter wave-lengths, and it therefore appeared likely that the absorbing substances in these two fractions were the allophanates of compounds having absorption maxima at 288 and 292 mμ; a hypothesis supported by the expected movement of these bands toward the near ultra-violet on alkaline hydrolysis. The hydrolysate of the lower fraction was re-allophanated and the maximum was observed to return to 279 mμ. This substance is being further investigated.

Since the tocopherols of the oily fraction from the original chromatographic absorption seem to be susceptible of allophanation it seems certain either that the hydroxyl groups are free in the original oil, or that they are in combination so labile as to be broken by contact with alumina.

In view of the failure to isolate allophanates by direct treatment of the fraction containing unsaponified fat, it was decided to saponify before allophanation; the comparatively small bulk of the fractions from the column allowed of shorter and more careful treatment during hydrolysis than is possible in handling the larger quantities of the original oil.

It is always found that the upper and lower fractions are each contaminated with a small proportion of the other, so that it is advisable, though not essential, to readsorb the unsaponifiable material before allophanation. This readsorption serves in addition, to remove most of the sterol set free in the hydrolysis; the remainder being precipitated out, as far as possible, with digitonin.

From the material absorbing at 295 mμ, β-tocopherol allophanate was isolated. The orange-coloured oil resulting from the allophanation, dissolved in

light petroleum (B.P. 40–50°), deposited on cooling to 0° a voluminous crystalline precipitate which was free from oil, and which carried with it only a small proportion of colour. Solution of this precipitate in a small volume of cold acetone removed a minute amount of an insoluble flocculent material which was probably a mixture of tritisterol allophanates. The small amount of colour remaining in the acetone-soluble fraction was then removed with blood charcoal from boiling methyl alcoholic solution. The product was recrystallized from anhydrous methyl alcohol, in which it had a very satisfactory temperature coefficient of solubility. At first the substance separated as small, compact, spherical aggregates, but after three recrystallizations as glistening feathery masses of lath-like crystals identical in appearance with those described by Todd *et al.* [1937], M.P. 144·5–145·5°. On anaerobic hydrolysis the allophanate yielded a colourless oil showing, in alcohol or hexane, an ultra-violet absorption with a maximum at 295 mμ $E_{1\,cm}^{1\%}$ 82 ± 2; persistence 10.

From the lower fraction showing the absorption at 292 mμ, α-tocopherol allophanate was isolated. The flocculent material mentioned above was much more abundant in this fraction, separating along with the allophanate and a good deal of coloured matter from alcoholic solutions. Acetone was employed for the separation; the α-allophanate being moderately soluble and the flocculent material almost entirely insoluble. The same solvent was used for the subsequent recrystallizations, as it was found to keep the oily contaminants and colour in solution in a very satisfactory manner. The final product had M.P. 158·5–159·5°, and gave on hydrolysis a colourless oil which in hexane had selective ultra-violet absorption with the double maximum at 292 mμ and 298 mμ as shown in the curve published by Emerson *et al.* [1937] with $E_{1\,cm}^{1\%}$ 298 mμ 80 ± 2; persistence 10. In solution in ethyl alcohol the curve showed only a single peak at 292 mμ; $E_{1\,cm}^{1\%}$ 70 ± 2.

In contrast to the findings of Evans *et al.* [1936] since confirmed by other workers, the α-tocopherol allophanate isolated by the above process was found to show a small optical activity, $\alpha_D^{25°} = +4\cdot8$. As it is likely that the α-tocopherol of the original oil is optically active, becoming racemized under the somewhat drastic conditions of the usual saponification, this figure may represent an incomplete racemization under the milder conditions employed for the saponification of our concentrates.

From 2 kg. of wheat germ oil approximately 1 g. of α-tocopherol allophanate and 0·75 g. of β-tocopherol allophanate were isolated.

In a previous paper, from the results of film measurements we postulated the existence in the molecule of a third ring. This was incompatible with the evidence on structure provided by the recent work of Fernholz, Karrer and Todd, from which it seems certain that both α- and β-tocopherols consist essentially of trimethyl-coumaran or -chroman rings bearing hydrocarbon side chains. We have been considerably disturbed by the discrepancy, particularly because the examination of a tocopherol prepared by Dr A. R. Todd and his associates which Danielli recently reported, indicated the area of 30 sq. Å., apparently more compatible with the simpler ring structure.

We were alive to the possibility that some of the preparations previously examined in our laboratory might contain traces of impurities or oxidation products which might give misleading measurements indicating a larger area; and we therefore examined the allophanates and tocopherols described in this paper, having confidence in their purity. To our surprise we obtained values of about 60 sq. Å. for all four compounds. Samples of pure α- and β-tocopherol allophanates, obtained through the kind co-operation of Dr A. R. Todd also

gave measurements indicating areas of about 60 sq. Å. Dr Danielli then very kindly re-examined for us the synthetic compound 5-hydroxy-4:6:7-trimethyl-2-*n*-heptadecylcoumarone for which data had previously been reported by him in a footnote to the publication of Bergel, Jacob *et al.* [1938]. He obtained the value of 51 sq. Å. Hence it appears that the second coumaran or chroman ring is pulled down into the surface, increasing the area to that of a 2-ringed structure. The oxygen atom of these rings must possess a much greater attraction for water than has hitherto been suspected.

Reviewing the foregoing findings we are satisfied now that the film measurements are compatible with the types of formulae recently suggested.

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CCLIV. THE ISOLATION OF PROGESTERONE AND 3:20-*ALLO*PREGNANOLONE FROM OX ADRENALS

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THE preparation from the adrenal gland of lipoid extracts which produced oestrous and progestational effects in the uterus of the immature rabbit was reported by Engelhart [1930]. These findings were confirmed by Callow & Parkes [1936], who showed that the active principles could be separated by the method of Allen & Meyer [1933]. They suggested, in view of the structure of the substances already isolated from the adrenal cortex, that the material responsible for the progestational activity might be progesterone or a closely allied compound.

By the courtesy of Prof. T. Reichstein of Zurich a concentrate of ox adrenal gland, obtained from his work on the cortical hormone, was placed at the disposal of Dr A. S. Parkes and made available to the author. Bioassays, carried out by Dr A. S. Parkes, showed this material to have an activity equivalent to 0·5 mg. progesterone per g.

As a preliminary step in the isolation of the active principle the crude material was saponified by treatment with sodium methoxide in anhydrous ether at room temperature. While such a saponification was not necessarily complete it provided a convenient method for the removal of a large amount of inactive acidic material without the destruction of progestational activity.

The alkali-insoluble material was then separated into ketonic and non-ketonic fractions. The ketonic material, on partitioning between suitable solvents, gave a semicrystalline concentrate from which a small amount of 3:20-*allo*-pregnanolone was isolated. Purification of the remainder of the concentrate by alumina adsorption, followed by vacuum sublimation, gave a crystalline product "*X*", M.P. 165–166°, which was subsequently shown to be a complex of *allo*-pregnanolone and progesterone.

Fractionating the crude concentrate has thus resulted in the isolation of progesterone M.P. 121° (uncorr.), $[\alpha]_D^{29°} + 193°$, together with somewhat larger amounts of 3:20-*allo*pregnanolone M.P. 191–192° (uncorr.). Similar results have been obtained by Reichstein [1938] using comparable material and a preliminary report of the work has already appeared [Beall & Reichstein, 1938].

Bioassays carried out throughout the work showed that the activity of the original concentrate could be accounted for by its "*X*" content so that the greater part, if not all of the activity was due to progesterone.

EXPERIMENTAL

Melting points are uncorrected.
Micro-analyses were by Dr A. Schoeller, Berlin.

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Preparation of the initial concentrate

The original material was prepared by N. V. Organon, Oss., from 1800 kg. of whole ox adrenals, and the concentrate on partitioning between pentane and 30% methyl alcohol [Reichstein, 1936; 1937] gave a pentane-soluble fraction weighing 1300 g., part of which was used in the present investigation.

Cold saponification

150 ml. of 7·5% sodium methoxide in methyl alcohol were added to 300 g. of the crude pentane-soluble material in 2 l. of dry ether. After 4 hr. at room temperature the mixture was diluted with 1 l. of ether and washed once with 500 ml. of 15% ethyl alcohol, once with 500 ml. of *N* KOH containing 15% ethyl alcohol and then once with 400 ml. of water. The aqueous solutions were combined and extracted with ether, the combined ether extracts being washed and taken to dryness to give 142 g. of "non-sap." Acidification of the alkaline washings, followed by ether extraction, gave 143 g. of "sap." All the progestational activity of the original material was found in the "non-sap." fraction.

Separation of the ketones of the "non-sap."

In a preliminary experiment 9 g. of a "non-sap." fraction in 50 ml. methyl alcohol containing 1·5 ml. of acetic acid were allowed to react for 1·5 hr. with 3 g. of Girard reagent T at room temperature [Reichstein, 1936]. The solution was then poured into 150 g. of ice and water containing 90% of the theoretical amount of NaOH necessary to neutralize the acetic acid, so that the final concentration of alcohol was 25%. It was then extracted four times with ether, the ether being combined and extracted three times with 35 ml. of 25% methyl alcohol.

The aqueous phase was then acidified to Congo red with HCl and, after standing for 1 hr., was ether-extracted to give ketones (A 1) 413 mg. Further acidification of the residual aqueous phase with HCl (10% by volume) and subsequent ether-extraction after 1 hr. gave 32 mg. of additional ketones (A 2). These were combined with the (A 1) fraction to give ketones (A).

The ethereal solution containing the unreacted ketonic and non-ketonic material, after being washed and taken to dryness, was refluxed in 50 ml. methyl alcohol, containing 3 ml. of acetic acid, with 3 g. of Girard reagent T for 1 hr. and then the ketones and non-ketones were separated as before, giving 280 mg. of ketones (B).

Bioassays showed that while the ketones A possessed an activity comparable with that of the original concentrate, the ketones B were inactive when given at five times this dosage level. Therefore in subsequent work the total ketones of the "non-sap." were separated by refluxing with Girard reagent and subsequently fractionated by reaction in the cold with more of this reagent. In this way the 142 g. of "non-sap." from 300 g. of original concentrate gave 4·4 g. of ketones A.

Partition of ketones A

4·4 g. ketones A were dissolved in 140 ml. ethyl alcohol which was then diluted to 200 ml. with water (70% final alcohol conc.) and extracted five times with 100 ml. portions of light petroleum, the latter being extracted in turn three times with 45 ml. portions of 70% alcohol. The alcoholic solutions were combined and extracted five times with 75 ml. portions of benzene which, on being taken to dryness, gave a semicrystalline residue weighing 1·63 g. and containing 92% of active material.

Isolation of 3:20-allopregnanolone and X

The 1·63 g. of semicrystalline benzene-soluble material were dissolved in a minimal amount of ether, several volumes of light petroleum were added and, after standing in the ice box, 303 mg. of solid material were obtained. Crystallization from benzene-light petroleum gave crystals melting at 182–189° which on recrystallization from aqueous ethyl alcohol gave colourless leaflets melting at 191–192° subsequently identified as 3:20-*allo*pregnanolone.

The remainder of the benzene-soluble material (1·3 g.) was dissolved in 60 ml. of alcohol which was then diluted with 90 ml. of water (40% final alcohol conc.) and extracted five times with 100 ml. portions of light petroleum. A small amount of insoluble material was present and was kept in the aqueous phase. The combined light petroleum solutions, which contained all the activity, were taken to dryness and the residue was sublimed *in vacuo*. At 80°/0·05 mm. it gave a small amount of inactive light oil but at 120°/0·05 mm. a partially crystalline yellow sublimate, containing all the active material, was obtained. Attempted crystallization of this sublimate from aqueous alcohol gave 232 mg. of sticky crystals which, on washing with ether, gave 36 mg. of crystals, M.P. 164–165°, which were designated "*X*".

Attempts to obtain more "*X*" from the ether mother liquors by cooling to −80° were unsuccessful, so the residue on evaporation was dissolved in 20 ml. of a mixture of one part benzene and twenty parts light petroleum and run through a column containing 5 g. of active alumina. The column was washed repeatedly with light petroleum until the movement of the coloured zone ceased. The coloured and colourless portions of the column were separated and eluted with a mixture of boiling benzene and alcohol, both extracts being progestationally inactive. The residue from the filtrate, however, had an activity which was equivalent to one-fifth that of progesterone, i.e. was of the same order as that of crystalline "*X*".

In another experiment sublimation of the alumina filtrate (450 mg.) from 3·24 g. of benzene-soluble material, followed by crystallization from aqueous alcohol gave 132 mg. of "*X*", M.P. 162–166°.

Separation of "X" into progesterone and 3:20-allopregnanolone

"*X*", M.P. 164–165°, was recrystallized twice from aqueous alcohol without any change in M.P. Recrystallization of this material from light petroleum gave crystals, M.P. 167–169°, which, on recrystallization from aqueous acetone melted at 165–166°. "*X*" therefore appeared to be a single substance.

Acetylation (pyridine and acetic anhydride at 100°), of 20 mg. "*X*" gave 12 mg. of an acetate, which, recrystallized once from aqueous alcohol, gave crystals melting at 136–137°. These were saponified by refluxing for 15 min. with 10 ml. of 2% alcoholic KOH and on crystallizing from aqueous alcohol formed needles which, alone, or mixed with authentic 3:20:*allo*pregnanolone (M.P. 191–192°) melted at 191–192°.

155 mg. of "*X*" dissolved in 10 ml. of 90% alcohol were treated with a solution of 1·2 g. of digitonin in 10 ml. of 90% alcohol and, after refluxing the mixture for 1 hr. it was left at room temperature overnight and then filtered. The filtrate was concentrated *in vacuo* and ether-extracted, the ether being well washed with dilute acid, alkali and then water. The residue, on evaporation, was sublimed at 120°/0·05 mm. to give 46 mg. of solid. Crystallization of this from light petroleum gave 10 mg. of progesterone which had M.P. 121° alone, and

mixed with authentic progesterone (M.P. 127°) melted at 121–123°. $[\alpha]_D^{23°}+193°$ ($l = 0.5$, $c = 1.01\%$ in ethyl alcohol). Its activity, on bioassay, was similar to that of progesterone. (Found: C, 80·1; H, 9·64%. $C_{21}H_{30}O_2$ requires C, 80·2; H, 9·62%.)

Identification of 3:20-allopregnanolone

76 mg. of crude *allo*pregnanolone, M.P. 178–185°, were acetylated and the acetate (65 mg.) was recrystallized twice from aqueous alcohol to give 52 mg. of *allo*pregnanolone acetate (leaflets) which alone, or mixed with authentic *allo*pregnanolone acetate (M.P. 142–143°), melted at 142–143°.

Saponification of the acetate in ether with sodium methoxide for 1 hr. followed by recrystallization from aqueous alcohol gave 32 mg. of leaflets which, alone or mixed with an authentic specimen of *allo*pregnanolone (M.P. 191–192°), melted at 191–192°. (Found: C, 79·1; 79·1; H, 10·5; 10·5%. $C_{21}H_{34}O_2$ requires C, 79·2; H, 10·8%.)

30 mg. of this pure *allo*pregnanolone were dissolved in 2 ml. of 90% acetic acid and treated with a solution of 50 mg. of chromic acid in 2 ml. of 90% acetic acid [cf. Butenandt *et al.* 1934]. After 6 hr. at room temperature the mixture was diluted with water, filtered, washed and the precipitate, recrystallized from aqueous alcohol, gave 12 mg. of *allo*pregnandione (leaflets), which, alone or mixed with authentic *allo*pregnandione (M.P. 198–199°), melted at 198–199°.

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CCLV. ACTION OF ARSENATE IN GLYCOLYSIS

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THE activating effect of arsenate on glycolysis in alcoholic fermentation as well as in muscle brei and extracts has been known for a long time [Harden & Young, 1906; 1911; Meyerhof, 1921], and it was also known that this activation is due specifically to increased hexosediphosphate breakdown [Harden & Young, 1911; Meyerhof, 1918]. The assumption that the effect of the arsenate was to activate a hexosediphosphatase splitting hexosediphosphate into hexose and inorganic phosphate was early abandoned [Meyerhof, 1927; Raymond, 1928; Macfarlane, 1930; Harden, 1932] as it was shown that arsenate activation of hexosediphosphate breakdown could not be obtained in the presence of the phosphatase only, independently of increased fermentation. Shortly after, it was found in yeast juice as well as in muscle extract that the production of alcohol or lactic acid from dihydroxyacetonephosphate, the breakdown product of hexosediphosphate, was increased by arsenate as much as that from hexosediphosphate, and further that the oxidoreduction of triosephosphate and acetaldehyde or pyruvate as well as the dephosphorylation of phosphoglycerate (phosphopyruvate) proceeded very much faster in the presence of arsenate [Meyerhof, 1934; Meyerhof & Kiessling, 1935]. Several attempts were made to explain the increased dephosphorylation of phosphoglycerate in the presence of arsenate. Since phosphoglycerate was known to be dephosphorylated after conversion into phosphopyruvate by transfer of its phosphate to adenylic acid forming adenylpyrophosphate and pyruvate, it was thought that arsenate, by activating the enzyme adenylpyrophosphatase, might increase the production of adenylic acid from the pyrophosphate. However, Pett & Wynne [1934] and Schäffner & Krumey [1936] showed conclusively that all the phosphatases investigated with the possible exception of adenylpyrophosphatase were not activated but even inhibited by arsenate; Needham & Pillai [1937] later showed that with adenylpyrophosphatase also the activation was only very slight and could not in any case account for such very greatly increased production of phosphate as is usually observed with arsenate. The dephosphorylation of phosphoglycerate in the presence of arsenate and catalytic amounts of adenylic acid in yeast juice or muscle extract occurs as fast as the transfer of phosphate from phosphoglycerate to equivalent amounts of adenylic acid, and it was therefore supposed that in such extracts the adenylic acid is somehow constantly being regenerated, but Meyerhof & Kiessling [1936] could not find any evidence of this and postulated the formation and breakdown of a cozymasepyrophosphate.

The coupled esterification of adenylic acid with inorganic phosphate consequent upon the oxidation of triosephosphate in muscle extract had by now been demonstrated [Meyerhof *et al.* 1937, 2; Needham & Pillai, 1937]. Needham & Pillai found that if arsenate was present during the oxidation no accumulation of adenylpyrophosphate resulted and they concluded that arsenate inhibited the coupling of esterification and oxidation. This interpretation was advanced

by other workers also [Meyerhof, 1937; Meyerhof *et al.* 1937, 1; 1938, 1] who supposed that the rapid oxidation of triosephosphate could take place only in conjunction with the esterification of inorganic phosphate with adenylic acid, and that arsenate, by inhibiting the coupling of oxidation and esterification, enabled the former to go on at the same rate even in the absence of large amounts of adenylic acid and inorganic phosphate. This assumption, besides explaining the observed activation by arsenate of the reaction triosephosphate + pyruvate (or acetaldehyde), would also show why hexosediphosphate breakdown itself can be influenced by arsenate in still another way. In normal glycolysis, where glycogen or glucose is the substrate, the adenylpyrophosphate formed during the dephosphorylation of phosphopyruvate is immediately dephosphorylated by donating its phosphate to glycogen or glucose, quite apart from the action of the adenylpyrophosphatase which is considered to be much slower. The adenylic acid is thus continuously regenerated to dephosphorylate more and more phosphopyruvate. When hexosediphosphate is the substrate it cannot however serve as phosphate acceptor for adenylpyrophosphate. The coupled esterification of adenylic acid during oxidation of triosephosphate goes on in both cases, but when arsenate is present the adenylic acid is not esterified and is free to dephosphorylate phosphopyruvate so that the breakdown of hexosediphosphate proceeds readily. In the case of glucose or glycogen this mechanism is of less importance.

Shortly afterwards Pillai [1938] showed that phosphoglycerate could be rapidly dephosphorylated in long-dialysed extracts of acetone muscle powder in the presence of arsenate if catalytic amounts of adenylic acid, Mg and cozymase are present, a result somewhat different from that of Meyerhof *et al.* [1937, 1] who found that in their A and B protein enzyme system a trace of hexosediphosphate too was necessary. They also stated that if an excess of adenylpyrophosphate is added to the arsenate-activated system, the phosphopyruvate breaks down but not the adenylpyrophosphate.

Since the arsenate activates not only the breakdown of hexosediphosphate as a whole but also all the known intermediate reactions individually, it seemed possible that the inhibition of the coupled esterification observed might be more apparent than real and that the action of the arsenate on this reaction should be investigated more fully. The muscle extracts used for the dephosphorylation experiments with phosphoglycerate contained no adenylpyrophosphatase [Pillai, 1938], and since, besides Mg and cozymase, adenylic acid was indispensable, it seemed, assuming as usual that the phosphopyruvate was dephosphorylated by transferring phosphate to adenylic acid, obvious to conclude that the adenylpyrophosphate must constantly be breaking down and reforming in the presence of arsenate to enable this reaction to take place. The effect of the arsenate in inhibiting the coupling might therefore very well be due to the accelerated breakdown by some mechanism, other than adenylpyrophosphatase, of the adenylpyrophosphate that is formed. The experiments described below show that in fact this is quite probable and that in the presence of arsenate the adenylpyrophosphate readily breaks down if a trace of phosphoglycerate, fluoride and cozymase are added.

EXPERIMENTAL

Enzyme preparation

An aqueous extract of a muscle powder prepared by precipitating fresh rabbit muscle extract with acetone was used [Needham & Pillai, 1937]. The extract was dialysed in cellophane tubes for 1–4 days according to the needs of the experiment. The enzyme solution does not contain any adenylpyrophosphatase.

Chemical preparations. Besides the preparations described by Pillai [1938], synthetic phosphopyruvate was made by the method of Kiessling [1936].

Methods of estimation. The usual methods adopted were the same as those given before [Pillai, 1938]. Adenylic acid and adenylpyrophosphate were determined when necessary by the deaminase method of Parnas & Lutwak-Mann [1935]. Details of the procedure will be found elsewhere [Needham & Pillai, 1937].

Experimental procedure. For most of the experiments 2 ml. enzyme solution were diluted to 4–6 ml. with the several additions of substrates, coenzymes, fluoride, arsenate etc. The amounts of these additions are given along with the experimental results. 0·3 ml. 6% $NaHCO_3$ was used as buffer in most cases and the experiments were carried out in ordinary 25 ml. centrifuge tubes. Suitable controls were always done simultaneously. The time of experiment was 15–30 min. at 38° or at room temp. (20°). Trichloroacetic acid was used to precipitate the proteins and the P estimations were carried out after filtration through dry filter paper. In the creatinephosphate estimations centrifuging was found quicker and more satisfactory than filtration.

The results are expressed, unless otherwise stated, as mg. increase or decrease per 2 ml. extract.

Details of experiments

It has been suggested that arsenate increases the rate of oxidoreduction between triosephosphate and pyruvate because this reaction goes on rapidly only when accompanied by simultaneous esterification of adenylic acid, and arsenate, by breaking this coupling, allows the reaction to proceed independently of the esterification [Meyerhof *et al.* 1937, 1; 1938, 1]. The following table shows the yields of lactic acid obtained during the dismutation of triosephosphate + pyruvate in the presence and absence of adenylic acid and inorganic P. The enzyme solution was an extract dialysed 73 hr. and containing practically no inorganic P.

Exp. I. All the tubes, including the control, received 2 ml. enzyme solution, 0·6 ml. $M/6$ Na hexosediphosphate, 0·8 ml. $M/5$ Na pyruvate, 0·6 ml. 0·15% cozymase, 0·1 ml. 2·5% $MgCl_2, 6H_2O$, 0·15 ml. 0·4M NaF and 0·3 ml. 6% $NaHCO_3$.

	mg. lactic acid
Control	+2·2
+ phosphate (1 ml. 0·16 M)	+4·5
+ phosphate + adenylic acid (12 mg.)	+6·6

The adenylic acid was neutralized before addition and all tubes made up to 6 ml. with water. Time of exp. 15 min. at 38°.

Both inorganic P and adenylic acid give increased dismutation but even in their absence there is a fairly active reaction. The effect of adding P alone seemed peculiar, but the following exp. shows that even in the absence of adenylic acid esterification is taking place, though of a different nature.

Exp. II. All preliminary additions as in Exp. I, except that an extract dialysed only 25 hr. and therefore probably containing traces of adenylic acid was used. 30 min. at 38° C.

	mg. inorganic P
Control + phosphate (1 ml. 0·16 M)	− 1·01
+ phosphate + arsenate (0·15 ml. $M/10$)	+0·22

There is a considerable disappearance of inorganic phosphate when it is present during dismutation even in the absence of adenylic acid; this is inhibited by arsenate. The ester formed cannot be adenylpyrophosphate or cozymase-pyrophosphate as the amount of adenylic acid present in the 25 hr.-dialysed

extract must be very small, also only 0·15 mg. cozymase is added (0·5 ml. 0·15 % solution of 20% pure cozymase). The ester was not identified but it might be formed by esterification of some of the pyruvate, the resulting phosphopyruvate being dephosphorylated in the presence of arsenate and the traces of adenylic acid still present. In some later experiments where an extract dialysed for much longer was used the esterification was found to be less but still noticeable.

The above experiment seems to show that the effect of the arsenate might be to increase the breakdown of any ester formed rather than to inhibit the esterification itself since it is known that arsenate activates the breakdown of phosphopyruvate. The next experiment shows that this is very probably the case for the oxidoreduction of triosephosphate + pyruvate and the corresponding esterification of adenylic acid.

Exp. III. Besides the preliminary additions given in Exp. I all tubes received 12 mg. adenylic acid and 1 ml. 0·16 M phosphate. In no. 3 the arsenate was added at the beginning of the experiment, but in 4 only after 15 min. at 38° when it was kept at 38° for a further 15 min.

		mg. inorganic P		min. at 38°
1.	Control	− 2·08	- 2·16	15
2.	Control	—–	− 2·37	30
3.	+ arsenate (0·15 ml. M/10)	−0·07	+0·13	15
4.	+ arsenate	−0·29	−0·31	15 + 15

The effect of the arsenate added before or after esterification is the same. In no. 4 the esterification has taken place but arsenate added later breaks down all the adenylpyrophosphate formed so that there is no difference between nos. 3 and 4. Whether in the presence of arsenate the esterification takes place at all is impossible of direct proof.

In order to see how the activation of phosphate breakdown actually happens it was necessary to exclude once for all the possibility of arsenate activation of hexosediphosphatase, to make sure that the extra inorganic P in the presence of arsenate did not come from this source. Exp. IV shows that in the presence of NaF only, the arsenate considerably inhibits the production of inorganic P from hexosediphosphate. This however is due to the fact that the arsenate greatly activates the dismutation of triosephosphate and thus reduces the effective concentration of hexosediphosphate itself. With both NaF and iodo-acetate the inhibition is very much less but still noticeable.

Exp. IV. All tubes received 2 ml. extract, 0·6 ml. M/6 hexosediphosphate 0·1 ml. 2·5% $MgCl_2$, 0·1 ml. of 0·4 M NaF and 0·3 ml. 6% $NaHCO_3$. Made up to 4 ml. with additions. All 30 min. at 38°.

	mg. inorganic P		
Control	0·91	—	0·69
+ arsenate (0·1 ml. M/10)	0·37	—	0·45
+ iodoacetate (0·1 ml. M/10)	—	0·79	0·72
+ iodoacetate + arsenate	—	0·66	0·63

The next point was to test whether added adenylpyrophosphate would be broken down during the dismutation of hexosediphosphate + pyruvate in the presence of arsenate and the following experiment shows that this is indeed the case.

Exp. V. All preliminary additions as in Exp. I. 15 min. at 38°.

	mg. inorganic P
Control	− 0·04
+ A.T.P. (0·8 mg. pyro-P)	+0·01
+ arsenate	+0·33
+ arsenate + A.T.P.	+1·19

In the absence of arsenate the adenylpyrophosphate does not break down.

It seemed therefore as if the oxidoreduction itself or one of the products formed could, in the presence of arsenate, cause adenylpyrophosphate to split off inorganic P. In accordance with this assumption the dismutation of two triosephosphate molecules alone without pyruvate could bring about decomposition of A.T.P., as is shown in the following experiment which was made with all additions as in Exp. V except for pyruvate which was omitted:

	mg. inorganic P
Control + arsenate	0·28
+ arsenate + A.T.P.	0·72

Since for the dephosphorylation of phosphopyruvate in muscle extract in the presence of arsenate and no adenylpyrophosphatase a small amount of adenylic acid acts catalytically and is indispensable, there is an obvious possibility that adenylpyrophosphate is continually being broken down and reformed. One would therefore expect that large amounts of adenylpyrophosphate should break down in extracts in which phosphopyruvate is at the same time experiencing decomposition. Addition of considerable amounts of adenylpyrophosphate to muscle extracts in which phosphoglycerate was being dephosphorylated in the presence of arsenate, however, failed to produce appreciable extra dephosphorylation.

Exp. VI. 0·5 ml. 0·1 M phosphoglycerate, 0·5 ml. 0·15% cozymase, 0·1 ml. $MgCl_2$, 0·2 ml. A.T.P. (0·1 mg. pyro-P) and 0·25 $M/50$ arsenate were added to 2 ml. muscle extract (dialysed 23 hr.) in the control and the other tubes. Made up to 5 ml. with 0·3 ml. $NaHCO_3$, water and other additions if any.

	Inorganic P	min. at 38°
	1·54	30
Control	1·60	60
+ A.T.P. (0·6 mg. pyro-P)	1·68	30

The negative result in this case, however, might be due to the fact that we are here dealing with the same enzyme reaction in both cases (the dephosphorylation of A.T.P. and phosphoglycerate requiring the same enzyme), or because at the moment when all phosphoglycerate has been dephosphorylated the A.T.P. has still remained intact. The former is unlikely because practically all the phosphoglycerate has broken down in 30 min. The latter would follow from the fact that so long as any phosphoglycerate (phosphopyruvate) is present the adenylic acid formed by breakdown of A.T.P. would be immediately rephosphorylated. Addition of NaF would of course prevent the dephosphorylation of phosphoglycerate and the consequent regeneration of A.T.P. The following experiment in which this was done shows that under these conditions the adenylpyrophosphate is completely broken down [compare Meyerhof *et al.* 1937, 1].

Exp. VII. All additions as in Exp. VI except that 0·5 ml. 0·4 M NaF was added (conc. of NaF $M/28$).

	Inorganic P
Control	0·30
+ A.T.P. (0·6 mg. pyro-P)	0·86

That the adenylpyrophosphate has actually broken down and has not merely removed the inhibition of phosphoglycerate dephosphorylation by NaF [Runnström & Hemberg, 1937] was shown by precipitating the residual adenylpyrophosphate with Ba acetate and estimating NH_2-N by the method of Parnas & Lutwak-Mann [1935]. There was no A.T.P. left. Further proof of this is given

below in experiments with only a trace of phosphoglycerate, which could not provide more than a fraction of the inorganic P by its own decomposition.

It seemed therefore that in the mere presence of phosphoglycerate and the complete oxidoreduction enzymes with arsenate adenylpyrophosphate could break down. Obviously this reaction is at the basis of the dephosphorylation of phosphoglycerate observed [Pillai, 1938] in the same system. There a small amount of adenylpyrophosphate is able to act catalytically, for immediately on decomposition it is rephosphorylated by some of the phosphopyruvate and this would go on continuously till all the phosphoglycerate has been decomposed. In view of the fact that the mechanism of the coupled esterification of adenylic acid

triosephosphate + cozymase + adenylic acid + inorganic phosphate
$$\rightarrow \text{phosphoglycerate} + \text{reduced cozymase} + \text{A.T.P.,} \quad \dots (A)$$
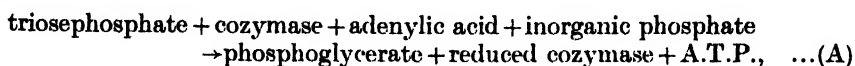
has been shown to be reversible [Meyerhof *et al.* 1938, 2]

A.T.P. + phosphoglycerate + reduced cozymase
$$\rightarrow \text{triosephosphate} + \text{cozymase} + \text{adenylic acid} + P, \quad \dots (B)$$
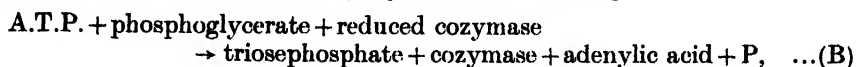
it was natural to suppose that the dephosphorylation of phosphoglycerate in the presence of traces of cozymase and A.T.P. was related to reaction B. This reaction is stoichiometric, but in the presence of alcohol or lactic acid to reduce the cozymase as soon as it is formed a small amount of cozymase can act catalytically. Leaving aside for a moment the mechanism of cozymase function one would suppose that part of the phosphoglycerate would undergo conversion into triosephosphate during its dephosphorylation. No accumulation of triosephosphate can however be expected, since triosephosphate in the presence of traces of cozymase and of the pyruvate formed by the dephosphorylation of phosphopyruvate would be reoxidized completely to phosphoglycerate when arsenate is present in the system [Adler & Günther, 1938]. As a matter of fact neither triosephosphate nor lactic acid could be detected among the products formed. However, if conversion into triosephosphate is concerned at all in the reaction one should get an inhibition of the dephosphorylation of phosphoglycerate if this change is prevented. By using synthetic phosphopyruvate instead of phosphoglycerate and employing NaF to prevent any conversion of the former into phosphoglycerate and then into triosephosphate, the dephosphorylation of phosphopyruvate should be considerably inhibited if the supposition is true. The following experiment in which this was done shows that the dephosphorylation of phosphopyruvate in the presence of traces of adenylic acid and cozymase is inhibited by NaF only slightly, both in presence as well as absence of arsenate.

Exp. VIII. 1·2 ml. Na phosphopyruvate containing 2·7 mg. phosphopyruvic P, 0·2 ml. A.T.P. containing 0·1 mg. pyro-P, 0·5 ml. 0·2% cozymase, 0·1 ml. 2·5% $MgCl_2$, $6H_2O$ and 0·3 ml. 6% $NaHCO_3$ were added to all tubes together with 2 ml. extract dialysed 23 hr. Made up to 5 ml. with further additions or water.

	Inorganic P	
Control	0·55	
+ NaF (0·3 ml. 0·4 M)	0·39	30 min. at 38°
+ arsenate (0·25 ml. $M/50$)	2·01	
+ arsenate + NaF	1·58	15 min. at 20°

The slight inhibition observed may be due to injury to the enzymes by the high concentration of NaF ($M/40$), or because, since only a trace of phospho-

glycerate is required for the reaction and the NaF poisoning is not 100 % effective, especially with large amounts of added phosphopyruvate, a small but sufficient amount of phosphoglycerate has formed in a short time. The following experiment in which decreasing amounts of phosphoglycerate are added in the presence of NaF shows that even with a trace of phosphoglycerate (0·12 mg. P) the adenylpyrophosphate is completely dephosphorylated.

Exp. IX. 0·5 ml. 0·2% cozymase, 0·1 ml. MgCl$_2$, 0·3 ml. M/50 arsenate, 0·6 ml. 0·4 M NaF and 0·3 ml. 6% NaHCO$_3$ were added to 2 ml. extract (dialysed 23 hr.) in all tubes including controls. Made up to 6 ml. with water or further additions. In each case a control containing the same amount of phosphoglycerate and a trace of A.T.P. was run to compensate for any dephosphorylation of the phosphoglycerate itself. 30 min. at 38°.

			mg. inorganic P
Control + 0·5 ml. M/5 phosphoglycerate	+ A.T.P. (0·75 mg. pyro-P)		− 0·66
+ 0·1 ml. M/5 ,,	+ A.T.P. (0·75 mg. pyro-P)		0·60
+ 0·5 ml. M/50 ,,	+ A.T.P. (0·83 mg. pyro-P)		0·87
+ 0·2 ml. M/50 ,,	+ A.T.P. (0·83 mg. pyro-P)		0·79

Practically all the pyrophosphate is broken down under these conditions and even at room temp. the reaction is quite rapid.

Exp. X. All additions were the same as in previous experiment except that 0·2 ml. M/50 phosphoglycerate and A.T.P. (1·1 mg. pyro-P) were added to all tubes. Reaction at room temp. (20°).

	Inorganic P
15 min.	0·69
30 ,,	0·84
60 ,,	0·99

It was observed that Mg is not necessary, but cozymase is indispensable and that iodoacetate completely inhibits the reaction. In the absence of NaF the dephosphorylation is very small, evidently owing to the disappearance of the phosphoglycerate by dephosphorylation after conversion into phosphopyruvate.

Exp. XI. All tubes received 2 ml. extract dialysed 77 hr., 0·2 ml. M/50 phosphoglycerate, 2 ml. A.T.P. containing 1·1 mg. pyro-P, 3 ml. M/50 arsenate and 0·3 ml. 6% NaHCO$_3$. Made up to 6 ml. with further additions or water. 0·5 ml. 0·2% cozymase, 0·1 ml. 2·5% MgCl$_2$, 6H$_2$O, 0·6 ml. 0·4 M NaF and 0·1 ml. M/10 iodoacetate added as indicated. 15 min. at room temp.

	Inorganic P
Control + NaF + Mg + cozymase	0·69
+ NaF + Mg only	0·46
+ NaF + cozymase only	0·68
+ cozymase + Mg (no NaF)	0·22
+ cozymase + Mg + NaF + iodoacetate	0·08

The dephosphorylation is quite high without cozymase but this is due to the fact that the adenylpyrophosphate used contains small amounts of cozymase as impurity. With adenylpyrophosphate freed from cozymase by leaving the solution overnight at pH 9·0, at which alkalinity the cozymase is inactivated, the indispensability of cozymase is quite clear. 15 min. at room temp.

	Inorganic P
No cozymase	0·07
+ cozymase	0·21

That no impurity in the sodium phosphoglycerate itself is responsible for the activation of adenylpyrophosphate breakdown can be shown by adding a small amount of synthetic phosphopyruvate to the extract, allowing it to remain

a few min. so that some of it is converted into phosphoglycerate and then adding the NaF, arsenate, coenzymes and A.T.P. This works as satisfactorily as the phosphoglycerate itself.

Sodium glycerate substituted for phosphoglycerate is completely inactive so that the possibility of decomposition of phosphoglycerate in such manner in the dephosphorylation system may be set aside. α-glycerophosphate and pyruvate have little effect. Even glyceraldehyde and pyruvate together did not influence the dephosphorylation of A.T.P. to any considerable extent beyond that by pyruvate itself.

Exp. XII. 2 ml. extract, 0·5 ml. cozymase, 0·1 ml. MgCl$_2$, 0·3 ml. $M/50$ arsenate, 1·7 ml. A.T.P. containing 1 mg. pyro-P and 0·3 ml. NaHCO$_3$ were mixed and made up to 6 ml. with water or further additions as follows: 0·4 ml. $M/5$ Na glycerate, 0·5 ml. $M/5\cdot5$ Na glycerophosphate, 0·5 ml. $M/5$ pyruvate, 0·5 $M/5$ glyceraldehyde and 0·6 ml. 0·4 M NaF. Time 30 min.

	Inorganic P	Temp.
Control + Na glycerate + NaF	+ 0·01	20°
+ glycerophosphate	+ 0·07	38°
+ glycerophosphate + NaF	+ 0·02	38°
+ pyruvate + NaF	+ 0·18	38°
+ pyruvate + glyceraldehyde	+ 0·31	38°

The reaction would therefore seem to be specific for phosphoglycerate.

In the absence of arsenate, A.T.P. is not decomposed in this enzyme system even in the presence of large amounts of phosphoglycerate and NaF, notwithstanding the fact that if no NaF is added phosphoglycerate itself will be dephosphorylated in such an extract with small amounts of A.T.P. added as coenzyme.

It is of interest to see whether creatinephosphate will be decomposed under the same conditions as adenylpyrophosphate in this system. The following experiment shows that in the presence of traces of phosphoglycerate and arsenate creatinephosphate will split off inorganic P even in the absence of adenylic acid. Mg is necessary for this, unlike the decomposition of A.T.P., and iodoacetate inhibits the reaction. Cozymase is indispensable.

Exp. XIII. 2 ml. extract dialysed 94 hr., 2 ml. creatinephosphate containing 1·2 mg. P, 0·2 ml. $M/50$ phosphoglycerate, 0·6 ml. 0·4 M NaF, 0·3 ml. $M/50$ arsenate, and 0·3 ml. 6% NaHCO$_3$ were mixed. Made up to about 6 ml. with further additions as indicated. 0·5 ml. 0·15% cozymase, 0·2 ml. 4% adenylic acid and 0·1 ml. 2·5% MgCl$_2$, 6H$_2$O. 30 min. at 20°.

	Inorganic P		
Control + Mg	0·22	—	—
+ Mg + cozymase	0·63	0·76	0·88
+ Mg + cozymase + adenylic acid	0·68	—	—
+ cozymase	—	—	0·49
+ cozymase + Mg + iodoacetate	—	—	0·10

It is curious that adenylic acid is unnecessary for the dephosphorylation of creatinephosphate. That the creatinephosphate itself contains no trace of adenylic acid as impurity can be shown by estimating the adenylic acid by the deaminase method of Parnas and also indirectly. It is known that adenylic acid is obligatory for the dephosphorylation of phosphoglycerate in the presence of arsenate and of the complete oxidoreduction enzymes [Pillai, 1938]. Creatinephosphate is also broken down in such an extract in the presence of phosphoglycerate. Even supposing that the mechanism of breakdown is the same in

both cases, if a small amount of creatinephosphate, which will be completely decomposed, is added to phosphoglycerate in the same extract with all additions except adenylic acid, one would expect some of the phosphoglycerate also to be dephosphorylated provided that the creatinephosphate contains some adenylic acid as impurity, but not otherwise. The following experiment shows that no extra phosphoglycerate is broken down if creatinephosphate alone is added, but the further addition of a trace of adenylic acid (0·1 mg.) immediately increases the production of inorganic P.

Exp. XIV. To 2 ml. extract dialysed 94 hr., 0·5 ml. $M/5$ phosphoglycerate, 0·5 ml. 0·15% cozymase, 0·1 ml. 2·5% $MgCl_2$, $6H_2O$, 0·25 ml. $M/50$ arsenate and 0·3 ml. 6% $NaHCO_3$ were added and made up to 5 ml. with water or further additions as follows: creatinephosphate (containing 0·6 mg. P) and 0·1 mg. adenylic acid when indicated. 30 min. at room temp.

	Inorganic P
Control	0·38
+ NaF	0·17
+ NaF + creatinephosphate	0·77
+ creatinephosphate	0·90
+ creatinephosphate + adenylic acid (0·1 mg.)	1·37

Further, adenylic acid does not activate the production of phosphate from creatinephosphate alone.

	Inorganic P
Creatinephosphate only (1·2 mg. P)	0·63
+ adenylic acid (0·8 mg.)	0·68

Creatinephosphate therefore seems to be able to split off inorganic phosphate in the presence of arsenate without the agency of adenylic acid or at most in the presence of extremely minute amounts.

It has already been mentioned that the mechanism of breakdown of A.T.P. in the presence of phosphoglycerate might possibly be related to the reaction phosphoglycerate + reduced cozymase + A.T.P. → triosephosphate + oxidized cozymase + adenylic acid + P which has been shown to occur in muscle extracts.

This requires reduced cozymase to start the reaction and since neither reduced cozymase nor any reducing agent such as lactic acid or acetaldehyde was added, it was thought possible that some material present in the extract itself might bring about this reduction.

Experiments in which considerable amounts of cozymase were added to the extract, either alone or in the presence of arsenate or with all further additions, failed to show the presence of any reduced cozymase when examined spectrophotometrically. Reduced coenzyme if present at all must be in very small amounts as the method detects amounts as low as 0·05 mg. reduced cozymase [Warburg *et al.* 1935].

DISCUSSION

The breakdown of adenylpyrophosphate by muscle extract containing no adenylpyrophosphatase, in the presence of arsenate, cozymase and small traces of phosphoglycerate (prevented from decomposition by NaF), has been demonstrated. This reaction is the basis of the dephosphorylation of phosphoglycerate in the presence of catalytic amounts of adenylic acid, observed under the same conditions in muscle extract [Pillai, 1938]. There the adenylic acid is esterified by phosphopyruvate and broken down continuously by the excess phosphoglycerate, and the reaction requires the presence of Mg, though adenylpyrophosphate itself seems to break down in its absence. Since Mg is obligatory for

the transfer of phosphate from phosphopyruvate to adenylic acid, it is easy to see that the dephosphorylation of phosphopyruvate proceeds via adenylic acid.

In the absence of arsenate, adenylpyrophosphate does not decompose even in the presence of very large amounts of phosphoglycerate and NaF, though phosphoglycerate itself under these conditions undergoes considerable dephosphorylation in the absence of NaF if a trace of adenylpyrophosphate is added. This shows that the mechanism of the reaction in which, in the absence of arsenate, phosphoglycerate is dephosphorylated in muscle extract containing no adenylpyrophosphatase and only traces of A.T.P. must be something entirely different from the reaction in the presence of arsenate as has been noticed before [Pillai, 1938].

It is difficult to understand the mechanism of the reaction by which adenylpyrophosphate is decomposed by muscle extract in the presence of traces of phosphoglycerate, cozymase and arsenate. It has been shown that the reaction

triosephosphate + cozymase + adenylic acid + phosphate
$$\rightleftharpoons \text{phosphoglycerate} + \text{reduced cozymase} + \text{A.T.P.}$$
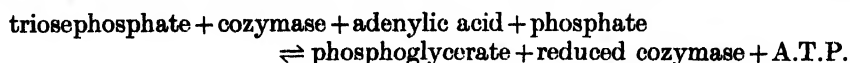
is reversible and can take place both in yeast and muscle extracts. The reaction is stoichiometric and the function of the A.T.P. in the reaction from right to left is to provide the energy by its breakdown to adenylic acid and inorganic phosphate for the conversion of phosphoglycerate into triosephosphate, an endothermic reaction. If lactic acid or alcohol is added to reduce the cozymase as soon as it is oxidized by the phosphoglycerate a small amount of cozymase can act catalytically. A small amount of A.T.P. would also suffice if some phosphate donator like creatinephosphate were added or the NaF omitted, so that some of the phosphopyruvate might itself rephosphorylate adenylic acid.

In the presence of arsenate not only does a trace of cozymase suffice but even a small amount of phosphoglycerate can act catalytically provided that NaF is added to prevent its decomposition to phosphate and pyruvic acid. It has been shown that the phosphoglycerate cannot act by directly splitting off phosphate since Na glycerate is inactive; also the inactivation by iodoacetate shows that the reaction involves some kind of oxidoreduction. Arguing from analogy with phosphoglycerate, glycerophosphate should be capable of reducing cozymase and being oxidized to triosephosphate in the same enzyme system but it has very little action upon the dephosphorylation of A.T.P. Similarly pyruvate, as well as pyruvate + glyceraldehyde, which can form a powerful oxidoreduction system with the formation of lactic acid, has comparatively little action. This breakdown of A.T.P. in the presence of arsenate, like the coupled esterification of adenylic acid, therefore seems to be more or less specific to phosphoglycerate ⇌ triosephosphate.

It will be observed that no reduced coenzyme could be found spectrophotometrically in the enzyme system producing dephosphorylation of A.T.P. even when large amounts of cozymase were added. The possibility of small amounts of reduced cozymase which cannot be detected by the analytical method employed being present must however be borne in mind.

That cozymase can act under certain circumstances as phosphate carrier must now be considered to have been proved beyond doubt [see Ohlmeyer & Ochoa, 1937] and it is likely that in all the reactions considered below a cozymasepyrophosphate is an intermediate compound, both in esterification and in liberation of inorganic phosphate from A.T.P. or creatinephosphate. The function of the adenylic acid, arguing from analogy with creatine, would then be to act as an acceptor of phosphate from the cozymasepyrophosphate, and of the A.T.P., like creatinephosphate, to be a donator of phosphate to cozymase.

Suppose now that a small amount of reduced cozymase is initially present to start the reaction from right to left in the reversible coupled reaction given above. It will be seen that very soon an equilibrium will be established, when as much triosephosphate will be oxidized by the cozymase to phosphoglycerate as phosphoglycerate is being reduced by the reduced coenzyme. No adenylpyrophosphate will however disappear in the sequel because an equivalent amount of inorganic phosphate will be esterified during the triosephosphate oxidation to replace the A.T.P. being broken down in the reverse reaction. To explain the actual breakdown of A.T.P. observed in the presence of the arsenate one would therefore have to assume that arsenate prevents the esterification of inorganic phosphate accompanying the oxidation of triosephosphate, without affecting the reverse reaction in any way, so that the equilibrium will be upset and the A.T.P. continually decomposed but not reformed. A small amount of phosphoglycerate and cozymase would then act catalytically, being alternately reduced and then oxidized. Of course the formation of an intermediate phosphorylated cozymase both in esterification as well as in dephosphorylation would not affect the final balance of the reaction.

However, this assumption of the inhibition by arsenate of the esterification of inorganic phosphate coupled with the oxidation of triosephosphate [Needham & Pillai, 1937; Meyerhof et al. 1938, 1] is difficult of experimental proof because the product of the oxidation of triosephosphate itself (phosphoglycerate) would cause any adenylpyrophosphate, even if it were formed, to break down immediately in the presence of the arsenate. No other similar reactions are known, so that this action of arsenate cannot be tested elsewhere. Arsenate as far as is known only slightly inhibits the phosphatases and does not prevent the transfer of phosphate from phosphopyruvate to adenylic acid, but these are rather different reactions, not comparable with esterification of inorganic phosphate. However, it seems unwarranted to assume in the absence of clear experimental evidence that arsenate inhibits the coupled esterification of inorganic phosphate, and the explanation of the breakdown of adenylpyrophosphate in the presence of arsenate has to await further knowledge.

It is known that arsenate activates the reaction triosephosphate + pyruvate (or acetaldehyde) → phosphoglycerate + lactic acid (or alcohol) to the same rate in the absence of stoichiometric amounts of adenylic acid and inorganic phosphate as when these are present in such amount. The supposition has been advanced that this is due to the fact that the reaction goes on rapidly only when accompanied by esterification of inorganic phosphate, and that arsenate, by breaking the coupling in some manner, allows it to proceed as rapidly even without simultaneous esterification. Why the reaction should go on rapidly, in the absence of large amounts of adenylic acid or inorganic phosphate, merely because the esterification is prevented by arsenate is difficult to understand. Without any assumption of inhibition by arsenate this activation can be explained by the fact that any ester formed (A.T.P. or cozymasepyrophosphate) is decomposed rapidly in the presence of phosphoglycerate and arsenate. Since there are always traces of coenzymes as well as inorganic phosphate present, the function of the arsenate would be to regenerate the coenzyme and inorganic phosphate by rapid breakdown of the coenzymepyrophosphate formed during the oxidation of triosephosphate, so that continuous esterification may occur. The arsenate thus assures a constant supply of coenzyme and inorganic phosphate for esterification and the reaction can go on at the same rate as when large amounts of adenylic acid and phosphate are present.

The behaviour of creatinephosphate is interesting. Since it can break down

in the absence of adenylic acid it is able to transfer phosphate directly to cozymase in the presence of arsenate, Mg and the complete oxidoreduction enzymes. Phosphopyruvate however is not dephosphorylated in the absence of adenylic acid, showing that it cannot transfer phosphate directly on to cozymase but only through adenylic acid, a result in keeping with other observed facts. Further, the necessity for the presence of Mg for the dephosphorylation of creatinephosphate but not for that of A.T.P. would indicate that the function of the Mg is to facilitate the transfer of phosphate between molecules of two dissimilar substances like creatinephosphate and cozymase and that its aid is not necessary for the exchange between two closely related compounds like adenylpyrophosphate and cozymase.

Finally, it has been observed that arsenate instead of activating sometimes inhibits glycolysis [Meyerhof, 1927], and also that it inhibits esterification of glucose [see Schäffner & Krumey, 1936]. While with the higher concentrations of arsenate the inhibition might to some extent be due to injury to the enzymes, it seems likely that the arsenate, by accelerating the breakdown of cozymase-pyrophosphate and adenylpyrophosphate, may prevent under certain conditions the esterification of carbohydrate by these sources and thus retard glycolysis as a whole.

SUMMARY

1. Adenylpyrophosphate breaks down rapidly into adenylic acid and inorganic P in the presence of arsenate in muscle extract containing no adenylpyrophosphatase if cozymase, a trace of phosphoglycerate and NaF (to prevent the decomposition of the phosphoglycerate) are added.

2. This reaction is the basis of the activation of hexosediphosphate breakdown by arsenate. The activation is prominent at two stages, (1) the oxidoreduction between triosephosphate and pyruvate (or acetaldehyde), and (2) the dephosphorylation of phosphoglycerate. The first reaction is considerably activated by the presence of stoichiometric amounts of adenylic acid and inorganic P which are esterified simultaneously, and since, in the presence of arsenate, the phosphoglycerate formed by oxidation of the triosephosphate causes a rapid breakdown of any A.T.P. formed, thus constantly regenerating adenylic acid and inorganic P for continuous esterification, the reaction goes on as quickly as before even when only traces of adenylic acid and inorganic P are available. The dephosphorylation of phosphoglycerate similarly proceeds as rapidly as when an equivalent amount of adenylic acid is present.

3. The mechanism of the reaction is not known but it is possible that it involves reduction of the phosphoglycerate to triosephosphate and formation of a cozymasepyrophosphate as an intermediate phosphorylated compound.

4. Creatinephosphate can break down similarly in the presence of arsenate, cozymase, Mg and a trace of phosphoglycerate and NaF, apparently without adenylic acid as intermediary phosphate transporter.

5. This activated breakdown in the presence of arsenate of adenylpyrophosphate, which is the most important phosphorylating intermediary, might by preventing esterification of carbohydrate account for the inhibition of glycolysis by arsenate sometimes observed.

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CCLVI. THE OXIDATION PRODUCTS OF THE UNSATURATED ACIDS OF LINSEED OIL

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GREEN & HILDITCH [1935] investigated the effects of various oxidizing agents on the unsaturated acids of linseed oil and found that the best yields of hydroxy-acids were obtained by the action of alkaline permanganate, though even with this the yields were low; from linoleic acid, 40 % tetrahydroxystearic acid was obtained whilst the yield of hexahydroxy-acids from linolenic acid was only 15 to 18 % of that theoretically possible. These authors also investigated the oxidation of pure α-linoleic acid and isolated from it 65 % of the theoretical yield of tetrahydroxy-acid when they worked under the conditions described by Hazura [1888] and 38 % when the conditions given by Lapworth & Mottram [1925] were used. β-Linoleic acid oxidized under Hazura's conditions furnished only 5 % of the tetrahydroxy-compound. They concluded that the α-form of linoleic acid is the one normally present in natural oils and that the β-compound is a complex mixture of transposition products, more readily degraded by oxidation than is the original α-acid.

In 1935 we published a preliminary communication on the oxidation of the acids of linseed oil by means of alkaline permanganate: the proportions of the various hydroxy-acids formed were determined and the presence of lactonic acids established amongst the degradation products.

The methods available for the analysis of linseed oil or of any oil containing more than two unsaturated acids are still far from satisfactory. The solid bromination products obtained on bromination of linoleic and linolenic acids represent only a small proportion of the total bromides, and bromine determinations have therefore to be carried out on the various fractions of the liquid bromides. Only if the amount of one of the three unsaturated acids present is known can the proportions of the other two be calculated from the i.v. Kaufmann's "thiocyanogen number" [Kaufmann & Keller, 1929], which has been used to determine the amounts of linoleic and linolenic acids, depends on arbitrary assumptions and in the hands of different observers gives very variable results[1] [cf. Gay, 1932]. The conversion of pure oleic acid into dihydroxystearic acid is, however, almost theoretical. Lapworth & Mottram [1925] found that, working with 1 % KMnO₄ in very dilute solutions, a 96 % yield of the dihydroxy-acid was obtained. They found, however, that when as much as 12 to 13 % linoleic acid was present with the oleic acid, the total precipitate containing both the di- and tetra-hydroxy-acids corresponded only with 96 % of the amount of hydroxy-acid theoretically obtainable. They add: "Judging from the results of experiments which the authors have made on the production of hydroxyketostearic acid, the yield of dihydroxystearic acid may fall off very rapidly if the proportion of acids more highly unsaturated than oleic acid exceeds a certain maximum." It is not, however, stated what conditions were used in these experiments on the forma-

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[1] Griffiths & Hilditch [1934] found the method quite satisfactory if sufficient care were taken to exclude all traces of moisture.

tion of the hydroxyketo-acid, but they almost certainly must have differed from those in which only the hydroxy-acids were produced.

In our experience using Rollett's [1909] conditions of oxidation, the conversion of oleic acid into dihydroxystearic acid is remarkably constant even in the presence of considerable quantities of the higher unsaturated acids.

15 g. acids obtained by the saponification of linseed oil were dissolved in 18 ml. 33 % KOH solution and 3 l. water added. The solution was cooled to 0° and 2 l. 1 % $KMnO_4$ solution previously cooled to 0° added during a period of 30 min., the mixture being constantly stirred and cooled with ice so that the temperature never rose above 5°; SO_2 was then immediately passed in until the MnO_2 was dissolved, and the solution acidified with dilute H_2SO_4. The precipitate, which at once separated, was filtered off, dried and extracted for 24 hr. with boiling light petroleum (B.P. 60–80°). The residue was then thoroughly extracted with boiling water to remove tetra- and hexa-hydroxystearic acids, dried and weighed. In three similar experiments it corresponded to 96 % of the dihydroxy-acid theoretically obtainable from the oleic acid present.

Two examples of crude oleic acid analysed in this way gave the following results.

(a) A commercial specimen, purified once by the Pb salt-alcohol method, I.V. 87·8. Two determinations were made, 5 g. acid being used for each. After allowing for the 0·95 g. saturated acid, the I.V. corresponded to 65 % oleic and 16·1 % linoleic acid. The percentage of oleic acid calculated from a 96 % yield of the dihydroxy-compound was 64·5.

Weight in g. of	(a)	(b)	Mean
Crude hydroxy-acid ppt.	5·14	5·24	5·19
Petrol-soluble saturated acids	0·88	1·01	0·95
Dihydroxy-acid	3·46	3·48	3·47
Tetrahydroxy-acid	0·34	0·32	0·33
Water-soluble residue	0·51	0·49	0·50

(b) A fraction of the unsaturated acids from butter which had been separated by distillation of the methyl esters and the unsaturated acids purified by the Pb salt-alcohol and Li salt-acetone processes gave 75·6 % oleic acid calculated from the I.V. and 75·4 % calculated from the dihydroxy-acid. Calculated from the I.V. 9·0 % linoleic acid was present.

The composition of the Calcutta linseed oil, I.V. 180, used for the oxidation experiments was investigated by the above method. After determining the proportions of saturated and oleic acids present, the amounts of linoleic and linolenic acids were calculated from the I.V. of the original mixture of acids.

	%
Saturated acids	10·90
Oleic acid	12·96
Linoleic acid	26·00
Linolenic acid	43·60
Unsaponifiable matter	1·20

Two determinations of the saturated acids, using the Pb salt-alcohol method of separation, were also made and gave 9·2 and 8·9 % saturated acids. When oxidizing larger quantities of material the best results were obtained if a preliminary separation of part of the saturated acid was first effected by applying the Pb salt-alcohol process. The unsaturated fraction was then worked up. Oxidation of 225 g. of unsaturated acids in 30 g. portions yielded hexahydroxy-acids corresponding to 39·9–51·6 % of the linolenic acid originally present and tetrahydroxy-acids corresponding to 27·6–32·3 % of the linoleic acid.

Table I. *Showing yields of oxidation products obtained from the oxidation of 225 g. linseed oil unsaturated acid fraction*

	(1) g.	(2) g.	M.P.
(1) Saturated acids	12·5	9·5	—
(2) Dihydroxystearic acid	36·0	38·2	132°
(3) Tetrahydroxystearic acid, soluble in boiling, insoluble in cold water	19·0	22·2	156–165°
(4) Hexahydroxystearic acids:			
(1) Linusic acid (obtained from aqueous extract after separation of the tetrahydroxy-acid) .	36·0	49·0	180–195°
(2) *iso*Linusic acid (obtained as oily ppt. on further concentrating aqueous filtrate from ppt. of hydroxy-acids. Purified by ether extraction)	18·8	20·8	173–175°
(5) Lactonic acid. $C_{12}H_{18}O_4$. Dark oil extracted by ether from *iso*linusic fraction	--	16·7	Oil
(6) Viscid yellow oil obtained by further concentrating aqueous filtrate. Contains lactonic acid $C_{12}H_{20}O_5$, small quantities of azelaic acid and other acids	--	75·2	Oil
(7) Soluble substances readily reducing $KMnO_4$. remaining in aqueous filtrate	—	--	—
(8) Volatile acids. Formic and n-hexoic present and probably propionic	—	—	—

It was estimated that fractions (1) to (6) contained approximately 80 % of the carbon of the acids oxidized.

The nature of the oil extracted by ether from the isolinusic acid fraction

This oil was not examined in our preliminary investigation.

After removal of the ether, a yellow limpid oil remained which gradually changed on standing in the desiccator to a dark brown viscous substance, sparingly soluble in ether, some form of polymerization possibly taking place. The oil was extracted with benzene and the portion soluble both in ether and in benzene examined.

	Analysis of fractions			Equivalent	
	% C	% H	I.V.	Neutralization	Saponification
(1) Benzene- and ether-insoluble	65·60	8·60	18·8	—	176·9
(2) Benzene-insoluble, ether-soluble	62·20	7·59	19·6	262·0	168·9
(3) Benzene- and ether-soluble	63·47	7·92	27·6	236·6	159·4
Theory for $C_{12}H_{18}O_4$	63·70	8·10	112·0	226·0	122·0

The clear yellow solution on standing deposited a brown viscous oil insoluble in benzene, so that polymerization possibly again took place in the benzene solution.

The difference between the equivalents obtained by direct titration and by saponification suggested the presence of a large proportion of lactonic acids. The analysis of the benzene-soluble oil agreed with the formula $C_{12}H_{18}O_4$; the difference between the neutralization and saponification values indicated the presence of the lactone of a hydroxy-dibasic acid. The actual determination of the end-point of the saponification value was difficult owing to the dark brown colour of the solution after boiling with alcoholic KOH. The discrepancy was, however, too big to be entirely explained in this way, and either the lactone ring was incompletely opened after 1 hr. boiling with alcoholic KOH or else some monobasic acid was present as impurity. The low I.V. obtained is typical of the

behaviour of the $\alpha\beta$-unsaturated acids. These data indicate that the constitution of the ether- and benzene-soluble oil is as follows:

$$OC.HC\!:\!CH.CH.(CH_2)_7.COOH.$$
$$\underset{O}{\underline{\qquad}}$$
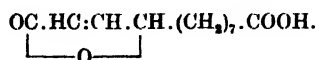
This would be derived from tetrahydroxystearic acid by the following reactions:

$$CH_3.(CH_2)_4.CHOH.CHOH.CH_2.CHOH.CHOH.(CH_2)_7.COOH,$$
$$CH_3.(CH_2)_4.COOH + COOH.CH_2.CHOH.CHOH.(CH_2)_7.COOH,$$
$$CH_3.(CH_2)_4.COOH + OC.CH\!:\!CH.CH.(CH_2)_7.COOH + H_2O.$$
$$\underset{O}{\underline{\qquad}}$$
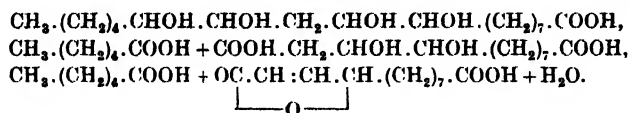
Evidence was subsequently obtained that the same lactonic acid was produced when pure linoleic acid was similarly oxidized. The very small proportion of azelaic acid isolated from these oxidation products seems to be explained by the tendency of the oxidized molecules to split between the 12th and 13th rather than between the 9th and 10th carbon atoms, leaving the comparatively stable lactonic acids.

Fraction obtained by further concentration of the aqueous solution after separation of the isolinusic fraction

After separating the *isolinusic* fraction, the solution was concentrated to a small bulk and cooled; a yellow viscid oil separated, from which, by extraction with limited quantities of hot water, 5 g. azelaic acid crystals were isolated.

Benzene-soluble fractions of the residual brown oil were analysed: C, 58·1, 58·8 %; H, 8·07, 8·18 %. The lactonic acid $C_{12}H_{20}O_5$ requires C, 59·01; H, 8·19 %. The percentage of carbon was considerably lower than that in the unsaturated C_{12} lactonic acid accompanying the *isolinusic* acid. Lactonic acids were, however, present in the oil since the equivalents determined by direct titration and by saponification were respectively 184·8–206·7 and 119–127. No more solid azelaic acid separated.

A further purification was effected by fractionation of the Na salts from solution in absolute alcohol. The dry salts obtained by neutralization with $N/2$ NaOH in the cold were extracted with absolute alcohol in which more than 50 % of the whole amount dissolved.

The alcohol-soluble Na salt contained C, 53·20; H, 7·26; Na 7·99 %. $C_{12}H_{19}O_5$ Na requires C, 52·16; H, 6·89; Na 8·33 %. This fraction appeared, therefore, to consist mainly of the Na salt of the γ-lactone of the 3:4-dihydroxydecane-dicarboxylic (1 : 10) acid $C_{12}H_{20}O_5$ represented above as an intermediate stage in the formation of the unsaturated γ-lactonic acid $C_{12}H_{18}O_4$. The existence of this lactonic acid suggested in our preliminary paper [1935] is therefore confirmed.

The alcohol-insoluble Na salt was recrystallized from dilute alcohol, the presence of a trace of water very greatly increasing its solubility. The percentage of Na varied from 10·1 to 15·83 in different fractions.

Originally we endeavoured to separate the constituents of this oily fraction by fractionation of the Zn salts. A small proportion of a Zn salt was obtained, the analysis and the equivalent of which agreed closely with those required by the formula $C_{12}H_{22}O_6Zn$ [1935]. In repeating this work, we again obtained a Zn salt which was, as before, less soluble in hot water than in cold and which, therefore, separated on warming its solution. We found, however, that on re-dissolving the salt in cold water and warming the solution, only a slight amount

was precipitated and it did not behave as a homogeneous substance. On acidifying the solution, an oily acid separated which gradually deposited a small amount of crystals of azelaic acid. Possibly an unstable double salt of Zn azelaate with the Zn salt of a higher acid is formed. From the evidence now available it seems probable that this fraction contained the lactonic acid of the β-hydroxy-acid corresponding with the unsaturated lactonic acid described above. We cannot confirm the isolation of the Zn salt of the dihydroxydibasic acid: in the light of further experience, it seems probable that the free $\beta\gamma$-dihydroxydibasic acid would be at once converted into the corresponding lactonic acid.

The oxidation of linoleic acid

In the first experiment, 90 g. linoleic acid were obtained from the solid tetrabromide isolated from the bromination of the unsaturated acids of maize oil [Nicolet & Cox, 1922]. The i.v. was 150 and it contained about 9 % saturated acids. The procedure already described for the oxidation of the linseed oil acids was followed for the oxidation of the linoleic acid. After the precipitate containing the tetrahydroxystearic acid and the saturated acids had been separated, the solution was further concentrated until the approximate concentration at which the *iso*linusic fraction separated from the oxidation product of the linseed oil acids had been reached. An oil then separated, completely soluble in ether and amounting to 11·4 % of the original weight of linoleic acid taken. Further concentration resulted in the precipitation of 12·2 g. of an oil from which a small quantity of azelaic acid (1·2 %) was crystallized.

90 % of the oil corresponding in its water-solubility with the "*iso*linusic fraction" was extracted with benzene and possessed the following properties:

	% C	% H	I.V.	Neutralization	Saponification
				Equivalents	
Benzene-soluble oil	65·88	9·53	2·05	272	184
Corresponding fraction from linseed oil acids	63·47	7·92	27·6	236·6	159·4

The benzene-soluble oil contained, therefore, some compound with a higher percentage of carbon than that of the C_{12} lactonic acid.

Determination of hydroxyl group

The lactone was esterified and the hydroxyl value of the ethyl ester estimated by the Zerewitinoff method. 0·2841 g. ethyl ester gave 12·28 ml. CH_4 at N.T.P. This would correspond with the presence in the oil of 55 % of the dibasic acid $C_{12}H_{20}O_5$ formed by opening the lactone ring.

Reduction

(a) *By sodium amalgam.* After treating with sodium amalgam in faintly acid solution for 12 hr., micro-determinations of the C and H contents of the oil showed that the substance was unaltered. No readily reducible ethylene linkage was present in the molecule.

(b) *By platinum oxide and hydrogen.* 0·45 g. absorbed 25 ml. hydrogen (N.T.P.). The equiv. wt. of the lactonic acid is 226 and 226 g. oil had absorbed 12·55 l., i.e. 0·56 mol. hydrogen. This determination together with the low i.v. (2·03) suggested the presence of 55 % of a substance containing an $\alpha\beta$-ethylene linkage; the close agreement of this figure with that obtained in the determination of the

hydroxyl group suggested that 55% of the $\alpha\beta$-unsaturated lactonic acid was present together with 45% of some saturated non-hydroxylated substance containing a higher percentage of carbon. The product gave no precipitate with dinitrophenylhydrazine so that the presence of a keto-group could be excluded. Since our starting material was linoleic acid and the product was saturated, dioxidostearic acid presented itself as a possible constituent, as it is saturated and contains no hydroxyl group.

The carbon and hydrogen percentages and the neutralization and saponification equivalents of a mixture of 45% dioxidostearic acid with 55% of the lactone of hydroxy(3)-decenic-(1:2)-dicarboxylic acid (1 : 10) would be:

	% C	% H	Equivalent By neutral-ization	By saponifica-tion
Theory	66·07	9·04	264·7	202·5
Benzene-soluble oil	65·88	9·53	—	—
	65·55	9·57	272	184

These results appeared sufficiently near to justify the conclusion that the oil consisted of a mixture of these two substances. We endeavoured to confirm this by reducing the mixture with HI and isolating the completely reduced acids.

Reduction with HI in boiling acetic anhydride [Crowder & Anderson, 1932]. 0·7 g. oil was boiled for 3 hr. with HI (sp. gr. 1·7) and red phosphorus in acetic anhydride solution. From the reaction product treated according to the directions given, stearic acid, M.P. 68°, was isolated by extraction with light petroleum and its identity confirmed by mixed M.P. From the petroleum-insoluble fraction a very small quantity of glistening white crystals soluble in hot water was obtained: these melted at 122–124°. n-Decanedicarboxylic acid was synthesized from ω-bromo-undecanic acid, for a specimen of which we are indebted to Dr J. C. Smith; the dicarboxylic acid melted at 125–126°, and admixture with the specimen obtained by reduction of the lactonic acid gave no depression. The crystals gave no evidence of lactonic properties. The presence of a C_{18} compound and of a derivative of the C_{12} dicarboxylic acid was thus confirmed. 0·7 g. dioxidostearic acid similarly reduced yielded 0·22 g. stearic acid.

The lactonic acid $C_{12}H_{18}O_4$ obtained as an oxidation product of the mixed linseed oil acids has, therefore, been identified amongst the oxidation products of pure linoleic acid. The presence of dioxidostearic acid appears not to have been previously noticed among the products of oxidation of linoleic acid by alkaline permanganate, although it is formed when oxidation is carried out by perbenzoic acid [Green & Hilditch, 1935].

Proportion of linoleic acid recovered as tetrahydroxy-acid
under Rollett's conditions of oxidation

In the first experiment carried out 90 g. linoleic acid (I.V. 150) (from which the saturated acid had not been completely removed) were oxidized. From the I.V. the proportion of pure linoleic acid was 83·1%; the weight of pure acid oxidized was therefore 74·8 g.; 50 g. tetrahydroxy-acid were isolated—a yield of 53·8%. In a second experiment, 30 g. linoleic acid, I.V. 166·7, were oxidized and 18·1 g. tetrahydroxy-acid recovered, a yield of 52·8%. The percentages of linoleic acid degraded were therefore 46·2 and 47·2. Green & Hilditch [1935] found a 65% yield of the tetrahydroxy-acid using Hazura's [1888] conditions, and 38%

when the conditions described by Lapworth & Mottram [1925] were employed. As we only isolated 27·6–32·3 % tetrahydroxy-acid from the linseed oil mixed acids, we confirm Green and Hilditch's results that when the mixed unsaturated acids are oxidized, a far larger proportion of the tetrahydroxy-acid is broken down than when pure linoleic acid is oxidized. In view of the finding of these authors, that the oxidation products of β-linoleic acid are almost completely degraded to lower carbon compounds, possibly the effect of the presence of linolenic acid is to increase the isomerization of linoleic acid from the α- to the β-form.

Oxidation of linolenic acid

Two experiments were carried out using linolenic acid prepared from the solid hexabromide obtained in the bromination of the linseed oil acids. The I.V. was 258 corresponding to 94·1 % of pure linolenic acid. 15 g. quantities were used for the oxidations: the yields of linusic acids were respectively 10·7 and 8·3 % and of isolinusic 14·7 and 14·0, making a total percentage yield of hexahydroxy-acids of 25·4 and 22·3 %. Green and Hilditch give 18 % as their yield. When the pure linolenic acid was oxidized about 75% was degraded to compounds containing smaller numbers of carbon atoms, but when the mixed linseed oils were oxidized the proportion of linolenic acid broken down to lower carbon compounds was only 55–64 %. The linoleic acid in the mixture appears, therefore, to exercise a protective action on the linolenic acid.

CONCLUSIONS

1. The products of oxidation of the mixed acids of linseed oil by means of alkaline permanganate under Rollett's conditions have been investigated.

2. The proportion of the various oxidation products has been determined.

3. The 96 % yield of dihydroxy-acid obtained from oleic acid under these conditions was very little affected by the proportions of linoleic and linolenic acid present.

4. The estimation of oleic acid in a mixture of unsaturated acids is most conveniently made as dihydroxystearic acid.

5. The yields of tetrahydroxy-acids from the linoleic acid present in the mixed linseed oil acids were only 28–32 %: yields of 52–54 % were obtained when pure linoleic acid was similarly oxidized.

6. The yields of hexahydroxy-acids calculated on the linolenic acid present in the mixed acids were 40–52 %. When pure linolenic acid was similarly oxidized, only 22–25 % hexahydroxy-acid was separated. Linoleic acid is therefore more readily degraded in the presence of linolenic acid and exercises a protective action on the oxidation of the linolenic acid.

7. The greater part of the degradation products consists of γ-lactonic acids containing 12 carbon atoms. The presence of the acid $C_{12}H_{18}O_4$ has been established and its constitution determined as the γ-lactone of hydroxy-(3)-decenic (1:2)-dicarboxylic acid (1:10). The same acid has been shown to occur amongst the degradation products of pure linoleic acid.

8. Evidence of the existence of the γ-lactone of dihydroxy-(3:4)-decanedicarboxylic acid has been obtained.

9. The amount of azelaic acid formed on oxidation under the given conditions is very small (1·2 %).

10. Fission takes place more readily between the 12:13 carbon atoms carrying the hydroxyl groups than between those in the 9:10 positions.

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CCLVII. EXPERIMENTS ON THE CHEMOTHERAPY OF CANCER

II. THE EFFECT OF ALDEHYDES AND GLUCOSIDES

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IN a series of recent papers Strong [1932; 1934; 1935; 1936, 1, 2] recorded that the feeding of natural oil of gaultheria inhibited the growth of spontaneous tumours in mice. Synthetic methyl salicylate had no such effect. Strong [1938, 1] showed that the effective substance was in the low-boiling fraction of the oil, and later that it was probably heptaldehyde [Strong, 1938, 2]. For this reason a number of aldehydes and ketones have been tested for their inhibitory action on the growth of grafted tumours in mice. The more promising compounds, citral and phloroglucinol aldehyde, have been tested on spontaneous tumours in mice.

The ease with which glucose penetrates into tumour cells made it appear possible that glucosides might be preferentially absorbed by tumour tissue and for this reason some available glucosides have been administered to mice with grafted tumours. None of them appeared to be effective in inhibiting growth and they were not tested on mice with spontaneous tumours.

Experiments with the Crocker Sarcoma 180

Substances were tested on groups of 5 mice with Crocker Sarcoma 180 as previously described [Boyland, 1938]; all compounds were given by oral injection 5 or 6 times weekly. The dosing was commenced immediately after grafting of the tumours, which were grafted into pure strain mice of either the Strong A or Little Dilute Brown (dba) strains. The tumours were measured thrice weekly and their growth compared with that of control tumours, which were growing in the same strain of mice grafted at the same time from the same tumour. Figs. 1, 4 and 7 show typical growth curves for the Crocker Sarcoma 180 in Strong A, Dilute Brown, and in stock mice. In stock mice the rate of growth in different mice is so variable that it is almost impossible to show the effect of inhibitory substances unless large numbers of mice are used. It is for this reason that mice from pure lines have been used for all the experiments with grafted tumours which are described in this paper. The compounds tested were administered in doses which would not injure the mice. The positive effects appeared to be of two kinds indicated in Table I.

Retardation in the rate of growth of the tumour as compared with that of the controls is indicated by *R*.

Increase in the length of life of the animals without effect on the rate of growth is indicated by *L*.

When no indication is made in the table the tumours in the treated animals were not significantly different from the tumours in control mice.

Table I. *The effect of orally administered substances on the Crocker Sarcoma 180 in Dilute Brown or Strong A mice*

R = retardation of tumour growth. L = increase in length of life.

Substance	Dose administered daily mg.	Effect
Anisaldehyde	5	—
o-Chlorobenzaldehyde	5	—
m-Chlorobenzaldehyde	5	—
p-Chlorobenzaldehyde	5	—
Cinnamic aldehyde	5	—
Citral	10	R
3:4-Dimethoxybenzaldehyde	5	R
p-Dimethylaminobenzaldehyde	5	—
3:4-Diethoxybenzaldehyde	5	—
Gentisic aldehyde	4	—
Heptaldehyde	5	—
"	50	—
o-Hydroxybenzaldehyde (salicylaldehyde)	4	—
m-Hydroxybenzaldehyde	4	—
p-Hydroxybenzaldehyde	4	—
2-Hydroxy-6-*tert*-butylbenzaldehyde	5	—
o-Methoxybenzaldehyde	5	L
2-Naphthol-l-aldehyde	10	—
6-Nitro-3-hydroxybenzaldehyde	5	—
Octaldehyde	5	—
Phloroglucinaldehyde	5	R
Resorcaldehyde	4	.
3:4:5-Trimethoxybenzaldehyde	5	—
Valeraldehyde	10	—
Oils:		
Oil of Wintergreen (from *Betula lenta*)	10	—
Crude oil of Betula	20	—
Refined oil of Betula	20	··
Betulol	20	—
Glucosides:		
Aesculin	10	—
Amygdalin	10	—
Arbutin	20	L
Helicin	10	—
Salicin	10	L
Ketones:		
Acetophenone	10	—
Dimedone	5	—
Methylheptanone	10	R
Methylheptenone	10	—

Retardation of growth was obtained with citral, phloroglucinaldehyde and 3:4-dimethoxybenzaldehyde. The effect with phloroglucinaldehyde is shown by comparison of Figs. 1 and 3. Fig. 2 shows the growth of grafted tumours in mice treated with heptaldehyde. Other experiments with heptaldehyde also showed no inhibition of the growth of grafted tumours. The results with phloroglucinaldehyde and citral were of sufficient promise to make it seem worth while to test related compounds.

The prolongation of life observed in mice treated with o-methoxybenzaldehyde and salicin is illustrated in Figs. 5 and 6 as compared with the controls in Fig. 4. The effect may be due to antiseptic action of these compounds reducing the toxic effects of any contamination which may be present in the grafted tumour.

Controls for
Figs. 2 & 3
Fig. 1.

Mice treated with
50 mg. heptaldehyde
daily
Fig. 2.

Mice treated
with 5 mg.
phloroglucinaldehyde
daily
Fig. 3.

Days after grafting

Tumour size as sum of length and breadth in mm.

Controls
for Fig. 5
and Fig. 6
Fig. 4.

Mice treated with
o-methoxybenzaldehyde
Fig. 5.

Mice
treated with
salicin
Fig. 6.

Controls in
stock mice
Fig. 7.

Days after grafting

Tumour size as sum of length and breadth in mm.

Fig. 10.

Tumour size as sum of length and breadth in mm.

567 568 539 561

Fig. 11.

Tumour size as sum of length and breadth in mm.

562 548 ←10 days→

Figs. 1-3. Growth of Crocker Sarcoma 180 in Strong A mice. Fig. 7 in Dilute Brown mice.
Figs. 4-7. Growth of Crocker Sarcoma 180 in mice. Figs. 4-6 in Stock mice.
Fig. 8. Growth of spontaneous mammary tumours in mice treated with heptaldehyde (50 mg. per day).
Fig. 9. Growth of spontaneous mammary tumours in treated mice.
Fig. 10. Growth of spontaneous mammary tumours in mice treated with citral.
Fig. 11. Growth of spontaneous mammary tumours in mice treated with phloroglucinaldehyde.

Methylheptenone 544
Methylheptanone 540
Valeric aldehyde 437

Fig. 9.

Tumour size as sum of length and breadth in mm.

550 B 497 518 504

Fig. 8.

Tumour size as sum of length and breadth in mm.

Experiments with spontaneous mammary tumours

Some experiments were carried out on spontaneous mouse tumours under the same conditions as previously described [Boyland, 1938]. Unless otherwise stated the dose was that given in Table I. Fig. 8 shows the experiment with heptaldehyde. In the case of one mouse (497) treatment caused partial retrogression and in mice 504 and 518 retardation of growth. Methyl heptanone and methyl heptenone were each tried on one tumour (Fig. 9), and the latter produced a temporary retrogression when first administered. Valeraldehyde (Fig. 9) caused inhibition of tumour growth but the mouse did not survive the treatment very long.

Experiments with citral and phloroglucinaldehyde, which were the two most effective aldehydes tested with the Crocker Sarcoma, are shown in Figs. 10 and 11. Citral produced partial retrogression in two mice and inhibition of growth of the other two tumours used. Phloroglucinaldehyde inhibited one of the two tumours on which it was tried.

An experiment was carried out to determine the effect of heptaldehyde on growth in body wt. of normal young mice. The groups of 4 mice each of the average wt., 11·3 and 13·5 g. respectively, were weighed daily. In one group each mouse was given 50 mg. heptaldehyde daily while the other group was left as controls. At the end of 20 days the average wt. of the first group was 19·5 g., while that of the second group was 20·5 g. The heptaldehyde did not affect body growth.

DISCUSSION

Of the series of aldehydes and ketones which have been tested, citral caused some inhibition of growth of both grafted and spontaneous tumours. Strong (personal communication) found that citral had some inhibitory action on spontaneous tumours in 1931. Heptaldehyde inhibited spontaneous tumours but not grafted tumours. The relative ineffectiveness of heptaldehyde in inhibiting grafted tumours while it inhibits spontaneous tumours to some extent is of interest in that its action is the converse of that of colchicine. Colchicine produces haemorrhage and some inhibition of grafted tumours but not of spontaneous tumours [Boyland & Boyland, 1937].

It is conceivable that the action of aldehydes is due to peroxide formation. Aldehydes are known to form peroxides on exposure to air and it is possible that different samples might contain varying amounts of organic peroxide. Maisin *et al.* [1938] have described the anticarcinogenic action of formaldehyde peroxides and the effect of heptaldehyde may conceivably be due to similar products.

SUMMARY

A number of aldehydes, ketones and glucosides have been tested for inhibitory action on tumour growth. Of these citral and heptaldehyde inhibited the growth of spontaneous tumours in mice.

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CCLVIII. OBSERVATIONS ON SPECIFIC NUTRITIONAL FACTORS IN LACTATION

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IT is well known that diet has a profound effect on lactation and that the satisfactory secretion of milk is only possible in the presence of certain known dietary factors in adequate quantities. There is also general agreement that a diet good enough to support the physiological strain of optimal lactation would also satisfy the requirements for optimal growth and reproduction.

The converse, however, does not seem to be necessarily true; in experiments on rats several authors have found that diets made up from natural foodstuffs which allowed, under stock colony conditions, of excellent growth and reproduction were very unsatisfactory with regard to the lactational performance of the animals [e.g. Smith & Anderson, 1929; Kon, 1931; Kon & Peskett, 1933; Coward, 1938]. Such findings obviously suggest the existence of separate dietary factors specifically connected with lactation, and publications to this effect are not lacking. A number of workers, who used so-called synthetic diets and included in them all dietary factors known by them to be required by the rat, have reported lactational failures, though otherwise the performance of the animals was very good. Efforts to find out the cause of the trouble have shown in several instances that this was nutritional and could be cured by supplying the rats with certain natural substances or with extracts from them which apparently contained a distinct new dietary principle capable of stimulating lactation. Thus, Mapson [1932; 1933] showed that a supposedly complete synthetic ration could be much improved as regards its value in lactation by the addition of fresh ox liver or of aqueous extracts thereof. It should be noted that liver exerted also a marked stimulating effect on the growth of young rats fed on the synthetic diet, but it is not known whether the two actions were due to the same or to different substances.

Similar observations on the favourable effect of liver in conjunction with semi-synthetic diets containing varying proportions of soya-beans were made by Wilkinson & Nelson [1931] and studies by Feaster & Nelson [1935] also showed the value of liver and led the authors to assume the existence of a new dietary lactational factor.

Nakahara and his colleagues [Nakahara & Inukai, 1933; 1934; Nakahara *et al.* 1934; 1935; 1936; 1937; 1938] have published a comprehensive series of papers in which they reported failure of lactation in rats kept on a presumably complete synthetic diet and marked improvement following the administration of fresh liver or of carefully fractionated liver extracts.

In addition to liver, other natural foods have been found to supply factors capable of stimulating lactation. Thus Tso [1927] and Outhouse [1937] found these present in lettuce, and the latter and also Meyer [1936] found them in

oats and to a lesser extent in hydrogenated cotton seed oil. It was shown that vitamin E was not involved.

Swanson & Nelson [1937], who used synthetic diets containing dried tinned pork muscle as the sole source of protein, were able to cut down considerably the number of lactational failures on such diets by the administration of fresh liver, but found another substance, the pancreatic hormone "lipocaic" of Dragstedt et al. [1936], to be extremely active in this respect.

These admittedly incomplete quotations from the literature show that many students of nutrition have had little success with their purified diets when lactational performance was considered, even though an effort was made to include in such diets all known essential food constituents.

When weighing up the evidence, however, the remarkable experiments of Cox & Imboden [1936, 1] should be placed on the other side of the scale. These authors fed in the course of 2 years, to three generations of rats, exclusively a conventional "synthetic" diet and obtained remarkable success in growth, reproduction and lactation, all of which were as satisfactory as on their good stock diet. A large group of female rats brought to maturity on the stock diet was put through 10 reproductive cycles on the synthetic diet and lactation in this case was also very satisfactory [Cox & Imboden, 1936, 2]. The diet consisted of casein, dextrin, lard, rice-cellulose and salts. The vitamin B complex was supplied as an aqueous, protein-free extract from yeast and the other addenda were wheat germ oil and an oily solution of carotene. Vitamin D was not supplied separately but it seems probable that the 9% of lard contained enough of the factor [Kon & Booth, 1934]. Richter et al. [1938] also noticed successful lactation when rats were offered a free selection of purified food constituents.

In planning our own investigations on the lactational performance of rats on purified diets we decided to use a diet similar to that of Nakahara & Inukai [1933] which has the advantage of simplicity and gave in the hands of these authors a high incidence of lactational failures.

EXPERIMENTAL

First series of experiments

Forty female rats (hooded Norwegian), born in the first half of April 1936, were reared on the stock colony diet until they were about 3 months old. This diet has the following composition:

Whole wheat, milled	69	Butter	5 ·
Linseed cake	14	CaCO$_3$	0·5
Crude casein	6	NaCl	0·5
Dried brewer's yeast	5		

In addition the stock rats receive fresh whole milk ad libitum, carrots or watercress daily, liver twice a week, about 5 g. each time. Pregnant and lactating females receive liver 3 times a week. The experimental rats were then placed on the following diet:

Ground rice (Patna polished)	75
Casein (New Zealand, repeatedly washed at the isoelectric point)	10
Butter (Empire)	10
Salts (Steenbock's 40)	5
Dried yeast (D.C.L.)	5

Table I. *Lactational performance of rats on the experimental (casein) diet and on the same diet supplemented with liver, liver extract and additional yeast*

Control group

Rat no.	1st lactation. Experimental diet only							2nd lactation. Experimental diet only						
	No. of pups				Av. wt. at weaning		Gain or loss in mother's wt. during lactation	No. of pups				Av. wt. at weaning		Gain or loss in mother's wt. during lactation
	Original		Weaned					Original		Weaned				
	♂	♀	♂	♀	♂	♀		♂	♀	♂	♀	♂	♀	
5328	4	4	4	2	42·7	39·0	+ 4	4	4	0	0	—	—	—
5351	5	3	4	3	39·2	38·7	− 7	3	2	3	2	45·0	41·0	0
5364	5	5	5	3	35·0	34·3	+ 2	3	6	1	1	35·0	29·0	+19
5397	3	5	3	5	40·7	41·4	+31	3 (+5)	0	3 (+5)	0	41·2	0	−13
5412	4	4	3	3	44·0	46·0	+12	3	5	3	5	39·0	37·4	+ 9
5427	4	4	4	4	44·5	41·7	− 8	—*	4	4	4	41·0	37·5	− 8
5444	4	4	4	4	42·2	39·5	−13	—†	4	—	—	—	—	—
5460	4	4	0	0	—	—	—	5	3	0	0	—	—	—
5464	4	4	0	0	—	—	—	4	3	0	0	—	—	—
5485	4	4	4	4	34·7	36·0	+ 5	4	4	4	4	43·2	42·2	+19
Average	4·1	3·9	3·1	2·8	40·4	35·1	+ 3·3	4·1	3·5	2·9	2·0	40·8	37·4	+ 4·3
% of pups weaned	75·6	71·8						69·7	57·1					
	73·7							63·4						

% of does lactating successfully: 80 75

Liver group

Rat no.	1st lactation. Experimental diet + liver							2nd lactation. Experimental diet only						
	No. of pups				Av. wt. at weaning		Gain or loss in mother's wt. during lactation	No. of pups				Av. wt. at weaning		Gain or loss in mother's wt. during lactation
	Original		Weaned					Original		Weaned				
	♂	♀	♂	♀	♂	♀		♂	♀	♂	♀	♂	♀	
5325	4	4	3	3	48·3	47·7	+14	3	5	3	5	44·3	42·2	+ 9
5388	4	4	3	3	44·2	41·7	+15	4	4	4	4	41·0	37·0	+19
5413	5	3	5	3	38·8	40·7	− 4	4	4	4	4	43·5	39·5	+ 2
5428	4	4	2	4	48·5	44·5	+20	3 (+1)	4	3 (+1)	4	40·0	40·0	− 3
5434	4	4	0	0	46·5	44·5	+14	5	2	0	2	50·2	46·0	−40
5437	—†	—	—	—	—	—	—	3	4 (+1)	3	0	—	—	—
5343	—‡	4	3	3	45·7	40·7	+19	—‡	—	—	—	—	—	—
5452	5	3	0	0	—	—	—	3	5	0	0	—	—	—
5471	4	4	3	4	44·7	40·7	+12	1 (+1)	1 (+5)	1 (+1)	1 (+4)	37·5	38·2	− 8
5484	4	4	3	4	44·7	40·7	+12							
Average	4·2	3·7	2·6	2·7	45·3	42·9	+12·9	3·5	4·4	2·6	3·0	42·8	40·5	− 3·5
% of pups weaned	63·2	73·5						75·0	68·6					
	68·4							71·8						

% of does lactating successfully: 78 75

Liver extract group

Doe	1st lactation. Experimental diet + liver extract							2nd lactation. Experimental diet only						
	No. of pups		No. weaned		Av. wt.		±	No. of pups		No. weaned		Av. wt.		±
5323	0	0	0	0	—	—	—	5	3	0	0	—	—	—
5352	4	4	0	2	32·0	34·5	+4	5	2	0	0	47·2	43·3	+17
5366	6	5	3	5	37·5	37·2	−11	3	5	5	3	—	—	—
5365	—†							5	2(+1)	0	0	—	—	—
5395	4	4	4	4	38·0	36·2	−5	5	4	4	1	45·2	42·0	+13
5436	4	4	4	3	38·5	35·7	−9	4	2	2	6	42·0	39·0	+5
5445	4	4	4	3	39·0	39·3	−3	6	4	4	4	47·5	—	+13
5459	4	4	0	0	—	—	—	4	2	0	0	—	—	—
5473	4	0	0	0	—	—	—	5	3	0	0	—	—	—
5478	4	4	4	4	39·0	34·2	−17	4	4	3	0	44·3	—	+6
Average	4·0	3·3	2·1	2·3	37·3	36·2	−6·8	4·6	3·3	1·8	1·4	45·3	41·9	+10·8
% of pups weaned	63·3	58·3						54·5	30·4					
	{60·8}							{42·5}						

% of does lactating successfully: 60·8 ... 67 (1st) 50 (2nd)

Yeast group

Doe	1st lactation. Experimental diet + yeast							2nd lactation. Experimental diet only						
	No. of pups		No. weaned		Av. wt.		±	No. of pups		No. weaned		Av. wt.		±
5321	4	2	3	2	42·0	39·7	+12	4	4	0	0	45·5	44·0	0
5350	4	0	2	3	—	36·0	+46	4	4	4	4	45·0	41·2	−10
5394	5	5	3	3	45·2	43·0	−2	3	3	4(+1)	4(+1)	40·7	41·3	−2
5396	4	3	4	4	42·0	40·5	−7	4	4	4	3	37·0	35·5	−41
5404	3	5	3	5	37·3	38·4	+16	3	5	3	4	—	—	—
5435	7	1	6	1	42·7	43·0	−10	6	2	—†		—	—	—
5443	4	4	4	0	—	—	—	—†				—	—	—
5451	2	3	3	0	—	40·7	+6	4	4	0	0	—	—	—
5472	3	3	3	2	40·7	40·0		2	0	0	0	—	—	—
5474	5	0	5	0	—	—	—	5	2	0	0	—	—	—
Average	4·2	3·5	2·2	2·0	41·7	40·1	+8·7	3·8	3·8	1·8	2·0	42·1	40·5	−13·3
% of pups weaned	52·4	57·1						46·7	53·3					
	{54·8}							{50·0}						

% of does lactating successfully: 54·8 ... 70 (1st) 50 (2nd)

After birth the no. of pups in the litter was reduced to 8, the 4 heaviest of each sex being kept. If the no. of live pups was under 8, substitutes were given when possible to make up this number.

* Resorption. † Doe not pregnant—too late to be remated. ‡ Died during pregnancy.

N.B. In the "no. of pups" column a + indicates substituted offspring.

This diet differs from that of the Japanese workers in that casein replaces an equivalent quantity of "fish protein". Mating was started after the does had been 1 week on the diet. Females in oestrus were paired with stock colony bucks, positive matings were detected on the following morning by the vaginal smear technique and inseminated does were placed at once under the various experimental treatments. The matings were completed within a week. The distribution of the rats was as follows: 10 does were kept on the experimental diet alone and formed the control group; 10 received, in addition to the diet, 1 g. daily of fresh beef liver (liver group); 10 were similarly given daily 1 ml. of liver extract equivalent to 5 g. of fresh liver (liver extract group). The extract was prepared by the method described by Nakahara *et al.* [1935] save that the preparation was stopped short at the fractionation by phosphotungstic acid. Finally, a last (yeast) group of 10 rats was given additional yeast to ensure that the vitamin B complex was not a limiting factor in the other diets. The yeast was supplied by mixing 90 parts of the basal diet with 10 parts of a potent brewer's yeast. On the 20th day of pregnancy the rats were placed in individual cages. If more numerous, the litters were reduced to 8 on the day of birth. In the case of smaller litters this number was, if possible, made up by using young from other mothers. The young rats were weaned at 21 days. All mothers were then transferred to the basal diet only and were kept on it to the end of the experiment. They were remated about 2 months after weaning and were allowed to produce and nurse a second litter. The results are given in Table I.

It is evident from the table that we were unable to repeat the results of the Japanese workers. Not only was lactation quite satisfactory on the basal diet alone, inasmuch as 8 out of 10 does nursed their young and 74% of the available young were weaned, but none of the additions tried (fresh liver, liver extract and yeast) improved their performance. If anything, the performance of the rats in the supplemented groups was below that of the controls.

With the second matings the results were very similar. This time the rats which had previously received fresh liver fared best, but there was little to choose between them and the group which was on the control diet throughout. The "liver extract" and "yeast" groups again lagged behind.

Second series of experiments

As casein was used in our diet instead of the "fish protein" fed by Nakahara & Inukai [1933], it was thought advisable to attempt to repeat the findings of these workers by using a diet as similar to theirs as possible. For this purpose white fish ("rock salmon") was obtained from a fishmonger. The muscle was freed from visible fat and skin and, after mincing, 10 lb. were boiled for 10 min. with 9 gal. of water containing a few drops of glacial acetic acid. The solids were allowed to settle, the supernatant liquid was poured off and the process was repeated with three further lots of water. On the last occasion the solids were strained off through muslin, dried in a hot air oven and milled. This protein was used in the experimental diet, which now had the same gross composition as that of Nakahara & Inukai [1933].

Twenty-six young does born in the stock colony towards the end of December 1936 were used for this experiment. They were placed at weaning on the experimental diet described on p. 1989 and 3 weeks later were given the fish protein diet. They were thereafter kept on this diet to the end of the experiment.

As our first aim was to find out whether we could obtain a satisfactory percentage of lactational failures on a synthetic diet, no curative or protective treatments were attempted, but all the available animals were put through

Table II. *Lactational performance of rats on the experimental (fish protein) diet*

Rat no.	1st lactation No. of pups Original ♂	Original ♀	Weaned ♂	Weaned ♀	Av. wt. at weaning ♂	Av. wt. at weaning ♀	Gain or loss in mother's wt. during lactation	2nd lactation No. of pups Original ♂	Original ♀	Weaned ♂	Weaned ♀	Av. wt. at weaning ♂	Av. wt. at weaning ♀	Gain or loss in mother's wt. during lactation
6041	0(+3)	2(+3)	3	2(+1)	42·7	41·3	+15	4	4	3	4	45·6	42·2	+20
6056	5	3	0	0	—	—	—	4	4	3	4	41·0	40·2	− 8
6065	2	6	2	3	56·0	53·3	+ 8	4	4	2	4	44·2	41·7	− 9
6068	4	4	0	0	—	—	—	5	4	0	0	—	—	—
6081	2	3	2	3	46·0	44·6	+10	5	3	2	1	29·0*	37·0	+12
6084	2	4	2	4	47·5	43·5	− 2	3	5	3	5	36·0	34·6	−14
6099	8	0	0	0	—	—	—	Some still-born						
6119	4	4	2	3	44·5	43·0	+28	3	5	3	5	37·3	36·0	+12
6142	4	4	4	4	41·2	39·5	+20	4	3	4	4	42·2	36·5	+11
6143	5	3	5	3	44·6	42·0	− 9	3	5	2	5	46·5	43·0	+26
6153	4	4	0	0	—	—	—	3(+1)	3(+1)	0	0	—	—	—
6159	4	4	4	3	37·5	30·3	+20	4	4	0	0	—	—	—
6162	4	4	0	4	—	—	—	5	4	0	0	—	—	—
6163	4	4	4	4	40·0	40·0	+14	5	1(+2)	2	1(+2)	46·0	48·0	+ 4
6173	4	4	4	4	44·2	44·2	− 4	4	3	4	3	44·7	44·3	+ 9
6174	1	7	1	6	40·0	42·1	+12	4	3	4	2	—	—	—
6183	5	3	0	0	—	—	—	1	3	2	2	50·0	53·0	− 6
6189	4	6	0	0	—	—	—	2	2	1	—	—	—	—
6190	0(+3)	1(+2)	0	0	51·5	45·6	+34	Not pregnant						
6197	0		2	3	—	—	—	4	4	4	4	41·5	41·0	+ 2
6212	0(+7)	0(+7)	0	0	—	—	—	Ate pups at birth						
6213	4	4	0	4	38·7	36·0	+30	4	4	4	3	35·5	38·6	− 2
6221	4	4	4	0	—	—	—	4	0(+4)	0	0(+4)	46·2	36·3	+12
6224	4	4	0	0	42·6	44·0	+22	2	3	4	0	—	—	—
6236	4	4	3	2	—	—	—	4	3	3	3	40·5	39·6	+13
6237	5	3	2		—	—	—	5	3	3	3	51·6	46·0	+14
Average	3·7	4·0	1·6	1·9	44·1	42·1	+14·1	3·8	3·6	2·2	2·5	42·4	41·1	+ 6·0
% of pups weaned	43·3	47·6						56·8	68·7					
	45·0							62·8						

% of does lactating successfully: 54   64

N.B. In the "no. of pups" column + indicates substituted offspring.

* One ♂ weighed at weaning only 20 g.

two reproductive cycles on the basal diet alone. Two consecutive matings were carried out in March and June 1937 and the results are given in Table II. The methods were as previously described.

This time again the results were not encouraging, the number of successful lactations being too high. Thus, 54% of the females at the first mating and 64% at the second mating reared young and 45 and 63% of the available young were weaned. Though the percentage of successful lactations in this experiment was a little lower than that usual in our stock colony (see Table IV below), the percentage of lactational failures was very much lower than that reported by Nakahara & Inukai [1933]. For reasons more fully set out in the discussion we do not believe that this basal diet could be used by us satisfactorily in the study of specific lactational factors.

Experiment involving hormonal treatment

An interesting point which emerges not only from this work but also from our stock colony records has been investigated further. We find, namely, that a female which fails to raise her young after her first parturition is more likely to fail after subsequent ones than a female which raises her first litter.

For example, in the first experiment (Table I) 7 rats failed twice, while out of 26 which raised first litters and produced second litters 21 weaned them successfully. Again, in the second experiment (Table II) 7 rats failed twice, but out of 14 which suckled once 13 were successful for the second time. It should be added, however, that a successful lactation may quite well follow an unsuccessful one, as for example in the second experiment where 4 rats reared pups from a second pregnancy, having failed to do so from the first one. Conversely, 2 rats which nursed their first litter normally failed to bring up the second. Such individual fluctuations in the lactational performance when the diet apparently remains unchanged have led us to suspect that the failure to rear young under normal (stock colony) conditions may be due to failure at some point of the hormonal mechanism now known to be involved in lactation [cf. Nelson, 1936; Folley, 1938]. In view of the importance of the anterior pituitary in lactation, it is reasonable to suspect that such failures might be due to lack of an anterior pituitary hormone. An attempt to explore this possibility was therefore made. As already mentioned, a certain proportion of the rats belonging to our stock colony fail to rear their litters. 21 such females, all of which had experienced their first parturition but had failed to rear their young, were used for an experiment on the possible connexion between lactational failures and the anterior pituitary hormones. In most cases living young were born to these females but all had died within a few days of birth. In three cases, marked with an asterisk in Table III, the young, as far as could be ascertained, were born dead, so that it is not really certain that the mother could not have reared them had they been alive.

The rats were divided at random into two groups and one group of 11 received on the day after their second parturition a subcutaneous implantation of one compressed tablet weighing about 80 mg. of acetone-dried and powdered pig anterior pituitary. The remaining 10 females were given no treatment.

The litters were treated at birth as already described on p. 1992 and the surviving young were weaned at 21 days. The results of the experiment are presented in Table III, from which it is evident that on this occasion the treated rats lactated no better than the controls. The marked incidence (over 75%) of failures confirms our view that female rats which fail once to rear their young are likely to fail again, but the experiment throws no light on the cause of the failures.

Table III. *Effect of one treatment with anterior pituitary powder on the ability to rear their second litters of rats which failed to rear their first litters on the stock diet*

Rat no.	No. of pups — Born ♂	Born ♀	Substitutes ♂	Substitutes ♀	Weaned ♂	Weaned ♀	Av. wt. at weaning ♂	Av. wt. at weaning ♀	Gain or loss in mother's wt. during lactation
									Treated rats
6390	5	5	—	—	4	4	41·7	42·5	+22
6480	1	3	—	—	1	3	52·0	48·0	−20
6562	4	6	—	—	0	0	—	—	—
6460	1	7	—	—	0	0	—	—	—
6438*	1	2	—	—	0	0	—	—	—
6463*	Some still-born		2	4	0	0	—	—	—
6527	1	1	—	2	0	0	—	—	—
6362*	1	0	—	5	0	0	—	—	—
6410	Some still-born		2	2	0	0	—	—	—
6590	4	4	—	—	0	0	—	—	—
6499	4	3	—	—	0	0	—	—	—
Av.	2·9	4·9	—	—	0·6	0·8	46·9	45·3	+ 1
% of pups weaned					19·2	15·9			

17·5

% of does lactating successfully: 18

Rat no.	Born ♂	Born ♀	Sub ♂	Sub ♀	Weaned ♂	Weaned ♀	Av wt ♂	Av wt ♀	Gain/loss
									Control rats
6513	8	5	—	—	7	0	43·5	—	+27
6420	6	2	—	—	4	2	49·5	47·5	+ 3
6422	0	3	4	—	0	0	—	—	—
6391	5	1	—	—	0	0	—	—	—
6579	Some still-born		3	3	0	0	—	—	—
6432	2	3	—	—	0	3	—	59·0	+ 4
6455	2	3	1	1	0	0	—	—	—
6447	2	5	—	—	0	0	—	—	—
6596	5	7	—	—	0	0	—	—	—
6445	6	1	—	1	0	0	—	—	—
Average	4·9	3·9	—	—	1·2	0·6	46·5	53·3	+11·3
% of pups weaned					25·0	14·3			

19·6

% of does lactating successfully: 30

* Only dead pups in first pregnancy.

N.B. Average of "pups born" includes substituted offspring.

Lactational performance of stock colony

The stock diet, the composition of which is given on p. 1989, was introduced into the laboratory in 1931 as the best of several diets tried and has been used ever since. This diet, containing a generous allowance of fresh liver, gives excellent fertility and growth. Nevertheless, a certain percentage of stock colony females fail to rear their young. A record of such failures for the years 1932–8 is set out in Table IV. It is hoped to present later a more detailed statistical analysis of the extensive data which we possess relating to the lactational performance of our stock colony rats. It will be seen that while in 1932 and 1933 some 90% of does weaned young, this percentage has been about 70% since 1934. The better performance in the first 2 years is possibly connected with more intensive breeding during that period, when the females were allowed only 2 weeks rest between weaning of young and next mating. Females are normally first mated at 3 months old and on those occasions when they were mated later

Table IV. Record of litters weaned and of litters lost in the stock colony for the years 1932–8

+ denotes weaned litters; – denotes that no pups were weaned

	1932 +	1932 –	1933 +	1933 –	1934 +	1934 –	1935 +	1935 –	1936 +	1936 –	1937 +	1937 –	1938 +	1938 –	% of failures 1932–8	Total litters	% of failures 1934–8	Total litters
Jan.	19	0	20	3	29	22	6	7	11	5	—	—	45	30	34·01	197	41·29	155
Feb.	10	2	25	3	8	8	—	—	2	1	12	14	—	1	33·72	86	52·17	46
March	16	1	5	2	11	8	24	12	2	2	41	10	10	16	31·88	160	35·30	136
April	17	1	38	1	1	8	1	0	22	7	6	5	31	6	19·44	144	29·89	87
May	12	4	46	3	8	7	—	—	29	4	18	7	56	17	19·91	211	15·75	146
June	21	2	—	—	31	7	29	8	—	—	30	8	11	0	17·01	147	18·55	124
July	10	5	21	1	4	0	4	6	—	—	—	—	0	4	20·09	55	55·56	18
Aug.	34	2	20	2	—	—	12	6	30	8	—	—	—	—	15·79	114	25·00	56
Sept.	8	1	31	8	32	5	11	4	3	0	73	36	—	—	25·47	212	27·44	164
Oct.	18	2	2	3	2	0	—	—	14	11	—	—	—	—	30·77	52	40·74	27
Nov.	1	2	—	—	21	7	2	1	—	—	—	—	—	—	29·41	34	25·81	31
Dec.	31	4	7	4	6	0	9	4	28	9	29	11	—	—	22·54	142	25·00	96
Totals	197	26	215	30	153	72	98	48	141	47	209	91	153	74	—	1554	—	1086
% of failures	11·66		12·77		32·00		32·88		25·00		30·33		32·60					

The high percentage of failures in Jan./Feb. 1934, Sept. 1937 and Jan. 1938 may possibly be due to the fact that the majority of rats used were 5 months old when first mated. Further, since 1933 the does have had longer rests between litters.

(Jan.-Feb. 1934, Sept. 1937 and Jan. 1938) there was a high percentage of failures.

A comparison of stock colony females and of the animals on the "synthetic" diets (Tables I, II and IV) shows that there is little difference between them regarding lactational performance if the criterion of successful lactation is the ability to rear young. Whether or not the synthetic diets used by us would prove in the long run adequate for growth and reproduction is a matter of conjecture. What remains certain is that the simplified diets satisfied, in these experiments at least, the lactational requirements of the nursing mothers almost as well as a stock diet of natural foodstuffs. It is significant that the stock diet supplies to lactating mothers over 1 g. of fresh liver daily and that the addition of such liver to the simplified diet was without effect.

The secretion of milk by rats which fail to rear litters

When litters are examined and weighed after birth it is our custom to see whether by gentle massage milk can be expressed from the mother's nipples. We have almost invariably been able to obtain a drop or two of milk in this way irrespective of whether the mother failed later to feed and rear her litter or reared it successfully.

DISCUSSION

In considering the question of dietary requirements for lactation, specific dietary lactational factors (if such exist) should be carefully distinguished from dietary constituents which are essential whether or not the animal is lactating and the requirements for which may be greatly increased during lactation. Specific dietary lactational factors may be defined as dietary constituents which are essential for lactation alone of physiological processes. In order to prove the existence of such specific "lactation vitamins" the first need, as Nakahara & Inukai [1933] recognized, is to discover a diet which, while allowing satisfactory growth and reproduction, causes complete failure of lactation without any other pathological effects. Our experiments, contrary to the findings of the Nakahara school, fail to provide any evidence of the existence of such dietary factors, since, on a diet which was as far as we could make it similar to the "Factor L"-deficient diet of the Japanese workers, a satisfactory proportion of our rats was able to suckle and wean two successive litters. It is of course possible that our diet might not prove so satisfactory over a longer period and it must further be borne in mind in connexion with our failure to confirm the results of Nakahara & Inukai [1933] that diets of the same gross composition often give varying results in different laboratories. Nakahara et al. [1938] have recently reported that rats experiencing their second lactations on the "Factor L"-deficient diet after having lactated once on a normal diet do not fail so signally to lactate as rats undergoing their first lactation on the "Factor L"-deficient diet. This would not explain our failure to confirm their original results, however, since we used rats experiencing their first lactation.

Incidentally, it is of interest to note that our experimental diet gave satisfactory growth, since the post-weaning growth rate of a group of rats fed solely on our version of the Japanese diet was as good as that of our stock colony rats (see Fig. 1).

The failure to rear their young which, as we have seen, is normally exhibited by about 30 % of our stock colony mothers, is probably not due to lack of specific dietary lactational factors, since milk can almost invariably be expressed from the teats of such rats. Nakahara & Inukai [1933; 1934], on the other hand,

reported that the mammae of rats which had failed to lactate on their "Factor L" deficient diet usually showed no signs of milk secretion on histological examination. We have not yet carried out histological studies on the
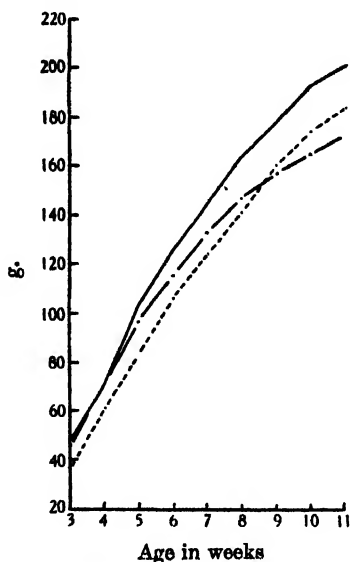
Fig. 1. Growth of female rats on the stock colony diet ·—· and ······· and on the experimental (fish protein) diet ——·—— Fish protein diet, December 1936. ·—· Stock diet, August 1936. ······· Stock diet, March 1937.

mammary glands of our stock colony mothers which fail to rear their young, but we believe that in such cases the failure to wean young may possibly be due to factors of a psychological nature. It is evident that failure to rear young may be due to other causes than lack of milk secretion. Thus Hain [1935] attributed the death of the young of oestrogen-treated rats to the unwillingness of the mother to allow the young to suckle rather than to inhibition of milk formation, while Daniels & Everson [1935] found that a high percentage of the young of rats fed on diets deficient in manganese died because they were too weak to suckle.

As far as they go, our experiments support the findings of Cox & Imboden [1936, 1, 2] that rats can lactate normally on simplified diets and we have not obtained any evidence of the existence of specific dietary factors stimulating lactation qualitatively different from the dietary principles normally required by the non-lactating rat.

SUMMARY

1. A simplified diet of ground rice, casein, butter, salts and yeast was found to produce satisfactory lactation in rats. Additions of fresh liver, liver extract or yeast were without effect on the lactational performance.

2. Fairly satisfactory lactation was also obtained on a similar diet in which casein was replaced by fish protein.

3. Stock colony rats which failed to rear their first litters were found to be more likely to lose their second litters than rats which successfully raised their first litters. Subcutaneous implantation of tablets of pig anterior pituitary into such rats just after the second parturition had no effect on their ability to rear their young.

Note added 29 October 1938. In view of the findings reported on p. 1992 concerning the effect of additional yeast it is of interest that Perla [1937] believes that an excess of vitamin B_1 has an untoward effect on lactation and the nursing instinct of rats.

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CCLIX. THE REDUCTION OF NITRATE TO AMMONIA BY *CLOSTRIDIUM WELCHII*

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From the Biochemical Laboratory, Cambridge

MANY bacteria are able to reduce nitrate to nitrite and this reaction has long been used in the identification and classification of these organisms. Quastel *et al.* [1925] showed that the reduction took place as a result of a coupled reaction with some H donor (e.g. lactate) and it was demonstrated that certain facultative anaerobes could grow anaerobically in the presence of nitrate on media which, in the absence of nitrate, could only support aerobic growth. The nitrate replaces oxygen as the final H acceptor [Quastel & Stephenson, 1925]. Green *et al.* [1934], working with toluene-treated *Bact. coli* and formate and lactate as H donors, concluded that the H transfer to nitrate takes place via a carrier; the nature of the natural carrier is so far unknown. Organisms possessing the hydrogenase enzyme [Stephenson & Stickland, 1931] as well as the nitrate-activating enzyme can utilize molecular H_2 as reducing agent.

Stickland [1931] made a detailed study of the reduction of nitrates by suspensions of *Bact. coli*. With lactate or molecular hydrogen as donor nitrate was reduced quantitatively to nitrite. With toluene-treated cells the H_2 uptake was also theoretical. There was no indication of any further reduction of nitrite with either plain or toluene-treated cells.

Reduction of nitrate to NH_3 has been obtained by Stocklasa [1908] with *Azotobacter chroococcum* and *radiobacter* and by Stocklasa & Vítek [1905] with four other organisms. In growth experiments, with *azotobacter* and *radiobacter* on an inorganic medium plus mannitol and nitrate, there was a disappearance of nitrate and formation of nitrite and NH_3 both aerobically and anaerobically. The effect was most marked in the case of *radiobacter* where, after 20 days, the inorganic N fraction consisted solely of NH_3; there was also considerable denitrification. With washed suspensions of *Bact. coli* and nitrate Aubel *et al.* [1937] found a small production of NH_3 in addition to some nitrite, when glucose was used as H donor.

In the course of a general investigation of the enzymic make up of washed cells of *Cl. welchii*, hydrogenase was detected and nitrate was found to be reduced in the presence of H_2. The H_2 uptake was greatly in excess of that required for the formation of nitrite. The work described in the present paper shows that nitrate, nitrite and hydroxylamine are reduced quantitatively to NH_3 by H_2 in the presence of washed suspensions of *Cl. welchii*. Similar results have been obtained with one strain of *Bact. coli*. Whilst this work was in progress a further paper by Aubel [1938] appeared, in which the quantitative reduction of nitrite to ammonia by suspensions of *Bact. coli* with glucose as H donor was demonstrated.

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Methods of estimation

(1) *Hydrogen.* H_2 uptakes were measured in Warburg manometers. CO_2 absorbers were placed in the inner tubes as the bacterial suspensions produced a little CO_2 when incubated. The main cup usually contained buffer and bacterial suspension and the substrate was tipped in from a side bulb after equilibration. The manometers were filled with H_2 or N_2 purified by passage over heated copper.

(2) *Ammonia.* NH_3 was determined on 1 or 2 ml. samples from the manometer vessels by the Conway & Byrne [1933] technique followed by nesslerization. Since NH_2OH ($\equiv 5\,\mu g$. N) produces a yellow opalescence and finally a dark precipitate in Nessler's reagent it was necessary to ascertain whether any distillation of NH_2OH occurred in the Conway apparatus. After distilling $56\,\mu g$. NH_2OH-N for 3 hr. at $37°$ the distillate gave no trace of reaction with Nessler's reagent, whilst $56\,\mu g$. NH_3-N were distilled quantitatively in 1 hr. at $37°$.

(3) *Nitrite.* Nitrite was estimated colorimetrically by the Griess-Ilosvay reagent as described by Stickland [1931]. Standard solutions of nitrite were checked by this method or volumetrically with $KMnO_4$.

(4) *Hydroxylamine.* Tests for NH_2OH were made and standard solutions checked by oxidation with I_2 to NO_2 and estimation of the latter colorimetrically [Endres & Kaufman, 1937].

The dry wt. of bacterial suspensions was estimated by means of a photoelectric turbidimeter [Clifton *et al.* 1935].

Unless otherwise stated all experiments were carried out at $37°$ and in phosphate buffer pH $7·1$ of final concentration $0·05-0·1$ M. To facilitate comparison quantitative data are all expressed in $\mu l.$, making the assumption, in the case of solids and liquids, that 1 g. mol. $\equiv 22·4$ l. Thus $1\,\mu g.$ NH_3-N, NO_3-N, NO_2-N or NH_2OH-N $\equiv 1·6\,\mu l.$ KNO_3 (B.D.H. "Analar") and $NaNO_2$ (Kahlbaum *pro analyse*) were used in this work. Hydroxylamine solutions were freshly prepared daily from the pure hydrochloride (Fraenkel and Landau) and neutralized to pH $7·1$.

Growth of organisms

The strain of *Cl. welchii* used in this work was that of the National Collection of Type Cultures No. 273 *Bacillus welchii S.R. 9* and isolated by Robertson from a fatal case of gas gangrene in 1914. Cultures of this organism have a tendency to "rope" and to form rough colonies; the preparation of suspensions is then difficult. The following procedure (Robertson, personal communication) was adopted for the maintenance of stock cultures. The organism was plated anaerobically on tryptic caseinogen broth-agar, a smooth colony picked off, and, after a few rapid subcultivations through Robertson's meat medium, sown into a number of tubes of alkaline egg medium. The latter were incubated 48 hr. anaerobically, sealed and stored in the dark at room temp. A working stock culture for sowing bulk cultures was maintained by daily subcultivation from one tube of Robertson's meat to another and incubation for 10–12 hr. anaerobically. After 7–12 such subcultivations the culture usually "roped" and a new subculture from the stock on alkaline egg was taken. Every 4–6 weeks the organism was replated and a new stock of alkaline egg cultures put up.

For the preparation of suspensions 900 ml. tryptic caseinogen digest broth pH $7·5$, containing a few pieces of meat from Robertson's meat medium, were autoclaved, cooled and sown at once with 1 ml. fluid from a 10 hr. culture on Robertson's meat medium. The culture was incubated at $37°$ in a McIntosh and

Fildes anaerobic jar for 10–12 hr. The following dry wt. determinations show that the growth obtained under these conditions is fairly uniform:

No. determinations	24
Mg. dry wt. organism/ml. culture:	
Range	0·15–0·27
Mean	0·21
Standard deviation	0·035

Longer incubation does not improve growth and the suspensions are less active. The culture was centrifuged, and the cells washed twice on the centrifuge with $M/50$ phosphate, pH 7·1; for washing the concentration of the organism should not exceed 1 mg. dry wt./ml., otherwise sedimentation is not sharp. The cells were finally suspended in water or buffer to give a concentration of *ca.* 10 mg. dry wt./ml. Addition of glucose to the culture medium gives even more rapid growth and greater bulk of organisms, but production of acid is very rapid and difficult to control and suspensions were less active from the point of view of the work in the present paper.

Two strains of *Bact. coli* have also been used: (*a*) *Escherichia coli* N.T.C. No. 86 (Strain I) and (*b*) a strain originating from the Bacteriological Department of Sheffield University (Strain II). Both were grown aerobically in flasks of tryptic caseinogen broth for 16 hr. at 37° and suspensions prepared in the usual way.

EXPERIMENTS WITH *CL. WELCHII*

The reduction of nitrate

The course of the H_2 uptake by suspensions of *Cl. welchii* in the presence of nitrate is shown in Fig. 1. There is a small blank H_2 output (rarely exceeding
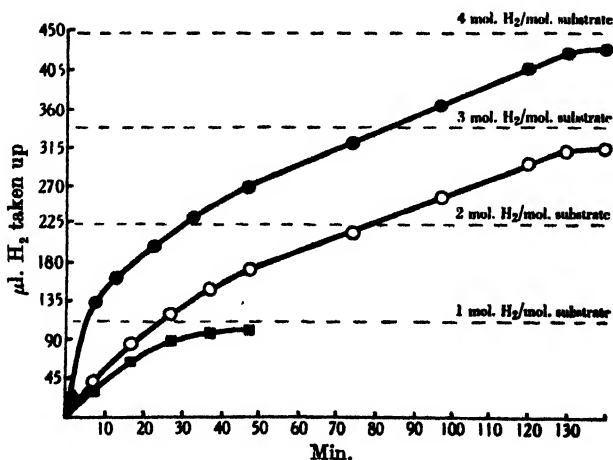
Fig. 1. Course of H_2 uptake. ● nitrate, ○ nitrite, ■ hydroxylamine. 1 ml. bacterial suspension (6·2 mg./ml.), 1 ml. $M/5$ phosphate buffer pH 7·1, 0·1 ml. $M/20$ substrate, 0·4 ml. water.

15 μl./hr.) with the organisms alone; a correction for this has been made. It will be seen that there is an initial rapid uptake of H_2 equivalent to about 1 mol. per mol. NO_3 followed by a slower uptake which continues until approx. 4 mol.

H_2 have been absorbed and then ceases abruptly. The reduction of nitrate to nitrite requires only 1 mol. H_2:

$$HNO_3 + H_2 = HNO_2 + H_2O, \qquad \ldots\ldots(1)$$
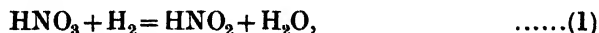
whilst an uptake of 4 mol. H_2 would correspond to a complete reduction to NH_3:

$$HNO_3 + 4H_2 = NH_3 + 3H_2O. \qquad \ldots\ldots(2)$$
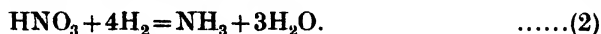
The presence of a volatile alkali giving the reaction with Nessler's reagent characteristic of NH_3 is readily demonstrated at the end of such an experiment. A number of experiments in which both H_2 uptake and NH_3 formation were estimated quantitatively have been carried out and the results of three such experiments are summarized in Table I. Controls were put up as follows: (a) without NO_3, (b) without H_2 (gas phase N_2), (c) without organisms. The quantitative data for the complete system NO_3-H_2-organism are in close agreement with the requirements of (2). The "H_2 uptake" in the N_2 gas phase experiments is due to the fact that there is a small H_2 evolution by the organisms alone in N_2 which is partially or completely suppressed in the presence of nitrate. It will be seen that there is also a small NH_3 production from nitrate in N_2; this production is always more than can be accounted for by the blank H_2 uptake in N_2 assuming that 4 mol. H_2 are required for formation of 1 mol. NH_3 (see last column $\frac{\mu l.\ H_2}{4}$ and cf. $\mu l.\ NH_3$ found). It would seem, therefore, that some unknown H donators, apart from H_2, are present in the cell which can also reduce NO_3 to NH_3. It will be shown later that this effect is more marked in the case of NO_2 and NH_2OH.

Table I

Theoretical values are those required by equation (2). The organism blank has been corrected for where necessary. 1 ml. bacterial suspension (*ca.* 10 mg./ml.), 1 ml. $M/5$ phosphate buffer pH 7·1, 0·1 ml. $M/20$ or 0·2 ml. $M/50$ KNO_3, water to 2·5 ml.

System	Exp. no.	Gas phase	H_2 uptake ($\mu l.$)			NH_3 production ($\mu l.$)			$\frac{\mu l.\ H_2\ found}{4}$
			Found	Calc.	% calc	Found	Calc.	% calc.	
$NO_3 + H_2 + Cl.$	1	H_2	330	358	92	91	89·5	102	83
welchii	2	H_2	443	448	99	115	112	102	111
	3	H_2	323	358	90	84	89·5	94	81
$NO_3 + Cl.$	1	N_2	9	358	2·5	6	89·5	6·8	2
welchii	2	N_2	4	448	1·0	6·5	112	5·9	1
	3	N_2	26	358	7·3	9	89·5	10·0	7
$NO_3 + H_2$	1	H_2	0	358	0	0	89·5	0	—

Table II summarizes the results of a number of experiments on the quantitative relationship between H_2 uptake, NH_3 production and original NO_3 and shows close agreement with equation (2).

Table II

All values corrected for organism blank

	mol. H_2 per mol. NO_3	mol. NH_3 per mol. NO_3	mol. H_2 per mol. NH_3
No. of determinations	18	11	11
Range	3·58–3·96	0·89–1·03	3·52–4·29
Mean	3·75	0·98	3·81
Standard deviation	0·111	0·057	0·224
Theory (equation 2)	4·0	1·0	4·0

The reduction of nitrite

The initial rapid uptake of 1 mol. H_2 that occurs in the reduction of NO_3 (see Fig. 1) suggested that reduction of NO_3 to NO_2 might be the first stage in the production of NH_3 from NO_3. In this case NO_2 should also be reduced when incubated with suspensions of *Cl. welchii* in presence of H_2. This was found to be the case; a steady H_2 uptake reaching almost 3 mol. H_2 per mol. NO_2 being obtained (see Fig. 1). This would correspond to the final reduction of NO_2 to NH_3:

$$HNO_2 + 3H_2 = NH_3 + 2H_2O. \qquad\qquad ......(3)$$

NH_3 was again found to be present and Table III gives the results of three quantitative experiments. The data for the complete system are in good agreement with equation (3). As with NO_3 the production of NH_3 in N_2 is greater than can be accounted for by the H_2 uptake in N_2. The effect is more marked than with NO_3 owing to the larger NH_3 production in N_2.

Table III

Theoretical values are those required by equation (3). The organism blank has been allowed for where necessary. 1 ml. bacterial suspension (*ca.* 10 mg./ml.), 1 ml. *M*/5 phosphate buffer pH 7·1, 0·1 ml. *M*/20 or 0·2 ml. *M*/50 NaNO$_2$, water to 2·5 ml.

System	Exp. no.	Gas phase	H_2 uptake (μl.) Found	Calc.	% calc.	NH_3 production (μl.) Found	Calc.	% calc.	μl. H_2 found / 3
NO$_3$+H$_2$+*Cl.* *welchii*	1	H$_2$	299	336	89	110	112	98	100
	2	H$_2$	255	269	95	85	89·5	95	85
	3	H$_2$	246	269	92	92	89·5	103	82
NO$_3$+*Cl.* *welchii*	1	N$_2$	3	336	0·9	12	112	10·4	1
	2	N$_2$	6	269	2·2	7	89·5	8·0	2
	3	N$_2$	41	269	15·2	33	89·5	37·0	14
NO$_2$+H$_2$	1	H$_2$	0	336	0	0	112	0	—

A number of experiments in which the quantitative relations between the various reactants of the complete system were determined are summarized in Table IV. The close agreement with equation (3) is apparent.

Table IV

All values corrected for organism blank

	mol. H_2 per mol. NO_2	mol. NH_3 per mol. NO_2	mol. H_2 per mol. NH_3
No. of determinations	11	6	6
Range	2·53–2·86	0·95–1·03	2·49–3·0
Mean	2·70	0·99	2·69
Standard deviation	0·112	0·036	0·193
Theory (equation 3)	3·0	1·0	3·0

The reduction of hydroxylamine

The possibility that NH_2OH might be an intermediate in the reduction of NO_3 and NO_2 to NH_3 was at once apparent.

$$HNO_3 + 3H_2 = NH_2OH + 2H_2O \qquad ......(4)$$
$$HNO_2 + 2H_2 = NH_2OH + H_2O \qquad ......(5)$$
$$NH_2OH + H_2 = NH_3 + H_2O. \qquad ......(6)$$
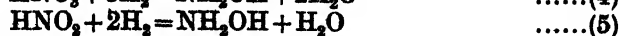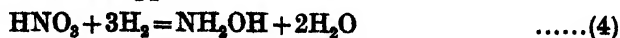
It was found that NH_2OH was rapidly reduced with an uptake of approx. 1 mol. H_2 per mol. NH_2OH (see Fig. 1), and NH_3 was found present at the end of the

experiment. The results of three quantitative experiments are given in Table V and are in good agreement with (6). The production of NH_3 in N_2 is larger than with either NO_3 or NO_2 and again this NH_3 formation cannot be accounted for by the H_2 uptake in N_2 (see columns 3 and 6). The greater magnitude of the effect in this case may be due to the same amount of endogenous H donators being able to reduce 3 or 4 times as much NH_2OH as NO_2 or NO_3 respectively; alternatively, these donators may be able to reduce NH_2OH more rapidly than the other substrates. The almost quantitative H_2 uptake shows that the reduction by H_2 takes place preferentially to the reduction by cell donators.

Table V

Theoretical values are those required by equation (6). The organism blank has been allowed for where necessary. 1 ml. bacterial suspension (*ca.* 10 mg./ml.), 1 ml. $M/5$ phosphate buffer pH 7·1, 0·1 ml. $M/20$ or 0·2 ml. $M/50$ NH_2OH, water to 2·5 ml.

System	Exp. no.	Gas phase	H_2 uptake (μl.)			NH_3 production (μl.)		
			Found	Calc.	% calc.	Found	Calc.	% calc.
$NH_2OH + H_2 +$	1	H_2	77	89·5	86	85	89·5	95·5
Cl. welchii	2	H_2	75	89·5	84	91	89·5	101
	3	H_2	96	112	86	106	112	96
$NH_2OH + Cl.$	1	N_2	2	89·5	2·2	18·5	89·5	20·7
welchii	2	N_2	6	89·5	6·7	37	89·5	40·8
	3	N_2	4	112	3·6	25	112	22·7
$NH_2OH + H_2$	1	H_2	0	89·5	0	0	89·5	0

The quantitative relationships between the initial and final products of the reaction in a number of determinations are summarized in Table VI and agree fairly well with equation (6).

Table VI

All values corrected for organism blank

	mol. H_2 per mol. NH_2OH	mol. NH_2 per mol. NH_2OH	mol. H_2 per mol. NH_2OH
No. of determinations	13	6	6
Range	0·81–0·95	0·85–1·01	0·83–1·10
Mean	0·88	0·94	0·92
Standard deviation	0·046	0·054	0·094
Theory (equation 6)	1·0	1·0	1·0

Evidence that nitrite is an intermediate in the reduction of nitrate

The initial rapid uptake of about 1 mol. H_2 during the reduction of NO_3 suggests that there is a preliminary formation of NO_2 which is then further reduced. It is significant that, after the initial burst, the succeeding slower rate of H_2 uptake is equal, within experimental error, to the rate of H_2 uptake with NO_2 (Fig. 1); this result has always been obtained in experiments in which the same batch of organisms has been used with NO_3 and NO_2.

The comparative rates of NH_3 production from NO_3 and NO_2 provide further evidence that NO_2 is an intermediate. The rate of H_2 uptake with NO_2 is slower than the initial rate with NO_3; taking the figures for the first 7 min. of the experiment of Fig. 1 and dividing by 3 or 4 to correct for the difference in total H_2 uptake the resulting figures give a measure of the maximum possible rate of NH_3 formation:

	μl. H_2	μl. $H_2/4$	μl. $H_2/3$
NO_3	133	33	—
NO_2	40	—	13

If NO_2 is an intermediate the slower reduction of NO_2 should control the overall rate of NH_3 production from NO_3 and the rates of NH_3 production from the two substances should be equal. Fig. 2 shows that this is the case. For these determinations a series of manometers were set up containing NO_3, NO_2 or NH_2OH and bacterial suspension in a gas phase of H_2. The course of the reaction was followed by the H_2 uptake and at intervals manometers were rapidly disconnected, the cups plunged into ice-salt mixture to stop the reaction and NH_3 estimated on 2 ml. samples. Controls without substrate were carried out.
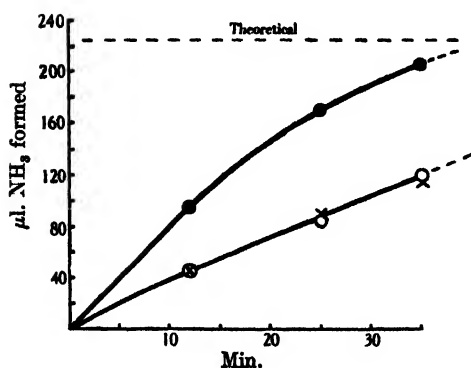
Fig. 2. Course of NH_3 production. × nitrate, o nitrite, • hydroxylamine. 1 ml. bacterial suspension (7·1 mg./ml.), 1 ml. $M/5$ phosphate buffer pH 7·1, 0·2 ml. $M/20$ substrate (224 μl.), 0·3 ml. water.
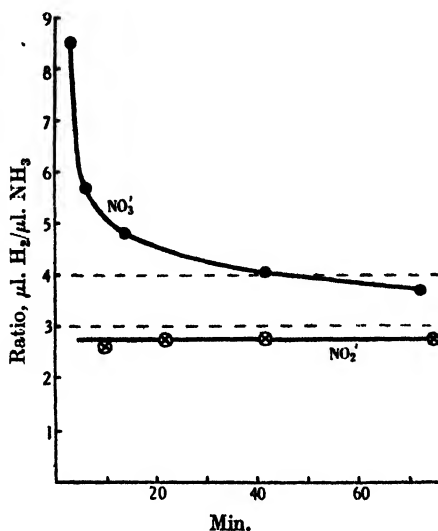
Fig. 3. Comparison of H_2 uptake with NH_3 production. 1 ml. bacterial suspension (10 mg./ml.), 1 ml. $M/5$ phosphate buffer pH 7·1, 0·2 ml. $M/20$ NO_3 or NO_2, 0·3 ml. water.

In Fig. 3 the ratio of H_2 uptake to NH_3 formation at various stages during the reduction of NO_3 is plotted. In order to obtain a more precise value for the H_2 uptake for the early samples it was necessary to stop the reaction by tipping in 0·2 ml. 10 % H_2SO_4 from a second side bulb as rapidly as possible after reading the manometer; even so the first two points may be up to 5 % lower than the true values. It will be seen that in the early stages the H_2 uptake is in excess of the 4 mol. required to reduce NO_3 to NH_3, but soon falls to approximately this value. The curve suggests that there may be an accumulation of a rapidly formed primary product of reduction (presumably NO_2) in the early stages. Experiments were carried out to test this possibility. A series of manometers containing NO_3 and *Cl. welchii* suspension in a H_2 gas phase were set up together with controls without NO_3. The NO_3 was tipped in from a side bulb after equilibration and the uptake of H_2 followed. At frequent intervals manometers were rapidly taken down and the cups plunged into ice-salt mixture. The contents were transferred to conical centrifuge tubes which were again cooled and then centrifuged at 0° till a sharp separation of the organisms was attained. 1 ml. of the clear supernatant was pipetted off (the organisms stir up easily) and used for nitrite

determination by the Griess-Ilosvay method. It was found impossible to use trichloroacetic acid as protein precipitant as there was a 20–30 % loss of NO_2 on acid treatment of quantities of the order found in these experiments. This loss was increased if the acid (though not the neutral) solution was filtered through kieselguhr.
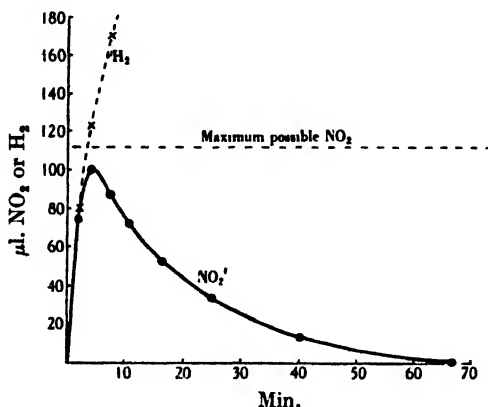
Fig. 4. Appearance and disappearance of nitrite. 1 ml. bacterial suspension (8·3 mg./ml.), 1 ml. $M/5$ phosphate buffer pH 7·1, 0·1 ml. $M/20$ KNO_3, 0·4 ml. water.

The curves given in Fig. 4 show the rapid production of NO_2 during the early stages and its removal as the reaction proceeds. The maximum NO_2 production reaches 89 % of that theoretically possible from the NO_3 added. During the period of rising NO_2 concentration the H_2 uptake is almost all accounted for by the NO_2 formed. Table VII shows that during the period of diminishing NO_2

Table VII

All values less organism blank

Time min.	$\mu l.$ H_2 absorbed (a)	$\mu l.$ NO_2 present	$\mu l.$ H_2 to convert NO_2 into NH_3 (b)	(a) + (b)
2	80	75	—	—
4	123	100	300	423
7·5	170	87·5	262	432
11	211	72·5	218	429
16·5	263	53	159	422
25	321	34	102	423
40	383	13·5	40	423
70	421	0	0	421

concentration the H_2 already absorbed plus the H_2 required to reduce the NO_2 present to NH_3 agrees with the total H_2 uptake of the reaction. Thus, both qualitatively and quantitatively, these experiments show that NO_2 is formed as an intermediate product during the reduction of NO_3 by H_2.

Evidence in favour of hydroxylamine as an intermediate in the reduction of nitrite

It has already been shown that *Cl. welchii* reduces hydroxylamine to NH_3 by H_2 and the question arose as to whether it may be formed as an intermediate during the reduction of NO_2 and NO_3 (see equations (5) and (6)). If this is so it is necessary that the rate of NH_3 formation from NH_2OH should be at least as great as that from NO_3 and NO_2 with the same batch of organisms. Reference to

Fig. 2 shows that this is so, the rate in this particular experiment being twice that with NO_3 and NO_2. In nine similar experiments with different batches of organisms the ratio rate NH_3 from NH_2OH/rate of NH_3 from NO_2 varied from 1·2 to 4·6 (six values lay between 2·2 and 3·3) but was never less than 1. From this point of view therefore NH_2OH satisfies the conditions for an intermediate.

If NH_2OH is an intermediate it will be reduced to NH_3 as rapidly as it is formed and the rate of reduction of NO_2 to NH_2OH will set a limit to the overall rate of NH_3 production from NO_2; there would therefore be little possibility of a direct detection of NH_2OH during the reaction. This view is supported by data on the ratio H_2 uptake/NH_3 produced at various stages during the reduction of NO_2. Fig. 3 shows that this ratio remains constant at just below 3 so that throughout the reaction the H_2 uptake is completely accounted for by the NH_3 formed.

Attempts were therefore made by the addition of ketonic substances to trap any NH_2OH which might be formed. So far no fixative has been found which forms a sufficiently stable oxime and at the same time is not itself attacked by the organism. Diacetyl completely inhibited the reduction of NH_2OH to NH_3 and also brought about a partial inhibition of NH_3 formation from NO_3 and a corresponding partial inhibition of the H_2 uptake. The results are rendered somewhat unsatisfactory by the fact that diacetyl itself is decomposed by the organism with uptake of H_2; large corrections have therefore to be applied to the H_2 uptake figures and the possible effect on the NO_3 reaction of reduction products of diacetyl is unknown. Oxaloacetate, pyruvate and α-ketoglutarate formed insufficiently stable oximes.

Summarizing the position as regards the possibility that NH_2OH is an intermediate, it must be emphasized that whilst all the data so far obtained are in accordance with such a view, there is no conclusive evidence of its truth.

Effects of concentration of substrate, pH and age of suspension on rate of reduction of NO_3, NO_2 and NH_2OH

Concentration of substrate. Table VIII gives the Q_{H_2} values obtained for the 5 min. period immediately after tipping in the substrate. The value for NO_3 gives an approximate idea of the rate of the preliminary reduction of NO_3 to NO_2 as the period measured is within the initial burst of the H_2 uptake (Fig. 1).

Table VIII

All values corrected for organism blank. 1 ml. bacterial suspension (6·2 mg./ml.) 1 ml. $M/5$ buffer pH 7·1, 0·1, 0·2 or 0·5 ml. $M/20$ substrate, water to 2·5 ml.

Conc. substrate	Initial Q_{H_2} values		
	NO_3	NO_2	NH_2OH
0·01 M	−349	−95	−99
0·004 M	−339	−77	−66
0·002 M	−211	−49	−41

It will be realized from these figures that most of the quantitative work already described has been carried out with suboptimal concentrations (0·001–0·002 M) of substrate. This was done in order to be able to measure total H_2 uptakes without adding more H_2 and re-equilibrating during the reaction.

The figures of Table VIII also give some idea of the absolute activity of the organism in terms of dry weight. Q_{H_2} values of this order have always been obtained with fresh suspensions of organisms and are comparable in magnitude with Q_{O_2} values obtained with aerobic bacteria.

Age of suspension. The effect of storage in N_2 at $0°$ on the initial Q_{H_2} obtained with NO_3, NO_2 and NH_2OH is shown in Table IX. It will be seen that the activity is relatively stable for 10 hr., falls off seriously in 24 hr. and has almost disappeared in 46 hr. The activity falls off slightly more rapidly with NO_3 than with NO_2 and NH_2OH.

Table IX

All values less organism blank. 1 ml. bacterial suspension (8·6 mg./ml.; 1·7 mg./ml. with NO_3). 1 ml. $M/5$ buffer 7·1, 0·4 $M/20$ NO_3 or NH_2OH or 0·2 ml. $M/20$ NO_2, water to 2·5 ml.

Age (hours)	Nitrate		Nitrite		Hydroxylamine	
	Q_{H_2}	% Q_{H_2} at 2 hr.	Q_{H_2}	% Q_{H_2} at 2 hr.	Q_{H_2}	% Q_{H_2} at 2 hr.
2	− 328	100	− 70	100	− 60	100
10	− 293	90	− 67	95	− 57	95
24	− 112	34	− 30	43	− 27	45
46	− 4	1	− 2	3	− 2	3

Effect of pH. The pH curve with NH_2OH (Fig. 5) was rather unusual in that the rate of reduction increased steadily with rising pH throughout the range of phosphate buffer. Controls without bacterial suspension showed that there was no purely chemical reduction of NH_2OH at any pH used.
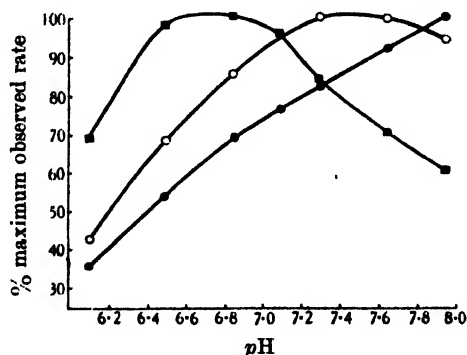
Fig. 5. Effect of pH. ○ nitrate, ■ nitrite, ● hydroxylamine.

The relation between pH and rate of H_2 uptake with NO_2 is also shown in Fig. 5. The rates plotted are those for the first 5 min. period since at pH 6·1 and 6·5 the H_2 uptake fell off sharply and soon stopped completely (Fig. 6). It appears that the organism is rapidly inactivated by nitrite at pH 6·5 and below. At pH 7·95, although the initial rate of H_2 uptake is slower than at pH 6·5, there is no inactivation and the reduction proceeds to completion.

Experiments on the effect of pH on the initial rate of H_2 uptake with NO_3 yielded a curious result. It was found that below pH 7·3 there is a marked lag period of about 5 min. before the rate becomes linear (Fig. 7). This lag period is less marked or absent at pH 7·3 and above. This phenomenon has been observed with two other batches of organisms and no explanation can be offered for it. In the pH curve for NO_3 in Fig. 5 the rates are those for the second 5 min. period after tipping by which time the rate had become linear at all pH values. As with nitrite the reaction stops short of completion at pH 6·1 and 6·5, although complete inactivation took longer to occur. Thus the H_2 uptake became reduced to 1/10 of the initial value after 25 min. at pH 6·1 and after 35 min. at pH 6·5. As it

has already been shown that NO_2 is formed faster than it is removed this effect may be ascribed to the accumulation of NO_2. All the above experiments with NO_3 were carried out with 1/5 the usual concentration of organism (*ca.* 2 mg./ml.)
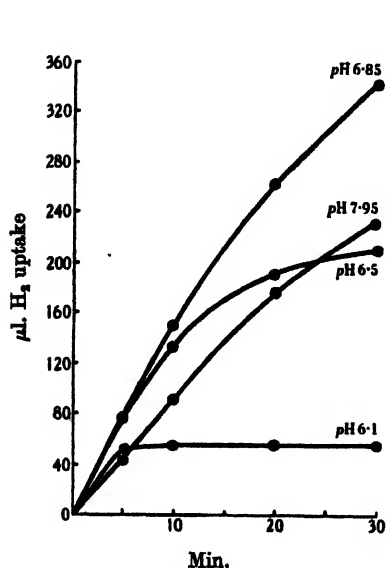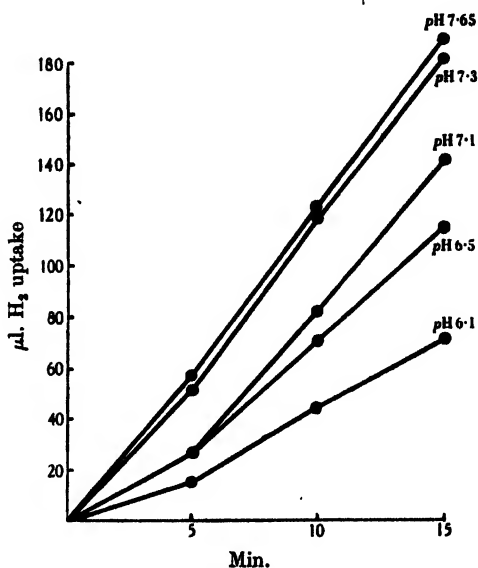
Fig. 6. Inactivation by nitrite at acid reaction. 1 ml. bacterial suspension (11·8 mg./ml.), 1 ml. $M/5$ phosphate buffer, 0·15 ml. $M/20$ $NaNO_2$, water to 2·5 ml.

Fig. 7. Lag period in H_2 uptake with nitrate. 1 ml. bacterial suspension (2 mg./ml.), 1 ml. $M/5$ phosphate buffer, 0·4 ml. $M/20$ KNO_3, water to 2·5 ml.

and a higher concentration of NO_3 ($M/100$) in order to confine the determinations to the initial rapid period during which the principal reaction is the reduction of NO_3 to NO_2.

EXPERIMENTS WITH *BACT. COLI*

Stickland [1931] has found that NO_3 is reduced quantitatively to NO_2 by H_2, in the presence of washed suspensions of *Bact. coli* (Esch.). His figures do not show any further reduction of NO_2. The chief experiments described above for *Cl. welchii* were repeated with two strains of *Bact. coli*; both reduced NO_3, NO_2 and NH_2OH to NH_3 in the presence of H_2 (Table XI), but quantitatively the rates of H_2 uptake with the various substrates varied greatly according to which strain was used (Table X). With strain II (see p. 2002) the rate of H_2 uptake, and

Table X

All values less organism blank. 1 ml. buffer pH 7·3, 0·1 $M/20$ substrate, 1 ml. bacterial suspension (exp. 1, 2·5 mg./ml.; exp. 2, 6·4 mg./ml.; exp. 3, 5·6 mg./ml.; exp. 4, 5·7 mg./ml.), water to 2·5 ml.

| Strain | Exp. no. | Initial Q_{H_2} | | |
		NO_3	NO_2	NH_2OH
Strain II	1	− 236	− 248	− 181
	2	− 242	− 253	− 115
Strain I	1	− 229	− 45	− 83
	2	− 113	− 6	− 39

therefore NH_3 formation, is greater with NO_2 than with NO_3. Thus, unlike the case of *Cl. welchii*, NO_2 would be removed as fast as it is formed and the limiting factor in the rate of reduction of NO_3 to NH_3 becomes the rate of the preliminary reduction to NO_2. The rate of H_2 absorption by NH_2OH is also rapid and it follows from the data of Table X and equations (2), (3) and (6) that the rate of reduction of NH_2OH to NH_3 is even greater than that of NO_2 (as with *Cl. welchii*).

Quite different results are obtained with Strain I. Here the data resemble those for *Cl. welchii* more closely (see Tables VIII and X) except that the rate of H_2 uptake with NO_2 is slower.

Table XI

Theoretical values are those required by equations (2), (3) or (6). All values corrected for the organism blank. 1 ml. bacterial suspension (*ca.* 5 mg./ml.), 1 ml. $M/5$ phosphate buffer pH 7·3, 0·1 or 0·2 ml. $M/20$ substrate, water to 2·5 ml.

Bact. coli	Substrate	Gas phase	H$_2$ uptake (μl.)			NH$_3$ production (μl.)			μl. H$_2$ found
			Found	Calc.	% calc.	Found	Calc.	% calc.	1, 3 or 4
Strain II	NO$_3$	H$_2$	435	448	97	111	112	99	109
		N$_2$	1	448	0·2	0	112	0	0
	NO$_2$	H$_2$	328	336	97	114	112	102	109
		N$_2$	2	336	0·6	5	112	4·5	1
	NH$_2$OH	H$_2$	97	112	87	112	112	100	97
		N$_2$	1	112	0·9	24	112	21·3	1
Strain I	NO$_3$*	H$_2$	308	896	34·4	38	224	17	—
		N$_2$	14	896	1·6	14	224	6·3	—
	NO$_2$*	H$_2$	102	672	15·2	37	224	17	—
		N$_2$	18	672	2·7	6	224	2·6	—
	NH$_2$OH	H$_2$	97	112	87	113	112	101	97
		N$_2$	0	112	0	33	112	30	0

* Not taken to completion.

The quantitative data presented in Table XI show that with Strain II the H_2 uptake and NH_3 formation with NO_3, NO_2 and NH_2OH are in conformity with equations (2), (3) and (6). This is also true of Strain I with NH_2OH. Owing to the very slow reduction of NO_2 by the latter strain the experiments could not be taken to completion but the production of NH_3 and uptake of H_2 are demonstrated. Table XI also shows that with both strains NH_3 production in N_2 is similar to that obtained with *Cl. welchii* and the same possible explanations are applicable. It has not been possible to determine whether the failure of Stickland's [1931] strain to carry the reduction further than NO_2 is due to difference of strain or to different conditions of growth—both of which factors in influencing enzyme production were at that time imperfectly appreciated.

DISCUSSION

The oxidations of NH_3 to NO_2 and NO_2 to NO_3 by the autotrophic soil organisms *nitrosomonas* and *nitrobacter* have long been known and considered as important stages in the circulation of N in nature. The reverse action—the reduction of NO_3 and NO_2 to NH_3—had until now only been demonstrated conclusively with *azotobacter* and *radiobacter* [Stocklasa, 1908] and for four other organisms [Stocklasa and Vítek, 1905]. The former are also soil organisms. The work reported in the present paper shows that such common organisms as *Cl. welchii* (one of the most widely distributed soil anaerobes) and some strains of *Bact.*

coli can also bring about this reduction energetically. It has been demonstrated that the reduction can be effected with the aid of molecular H_2; since *Cl. welchii* decomposes not only many carbohydrates but also some of the amino-acid constituents of tryptic caseinogen broth with evolution of H_2, a supply of this reducing agent would always be available. Furthermore, evidence was obtained that other unidentified H donators present in the cell can also bring about the reduction. Very recently Aubel [1938] has shown that washed suspensions of *Bact. coli* can reduce NO_2 (and therefore NO_3) to NH_3 using glucose as H donator. It is perhaps significant that glucose is fermented by *Bact. coli* with production of H_2. Lactate and succinate were unable to bring about the reduction of NO_2. Aubel also obtained some evidence that hyponitrite and hydroxylamine may be intermediates in the reduction of NO_2 by glucose with *Bact. coli*. The evidence is not very convincing as no data are given; it is simply stated "Il a été en outre possible de déceler des traces notables d'acide hyponitreux et d'hydroxylamine dans l'expérience *b*, en appliquant l'excellente technique de Lemoigne, Monguillon et Desvaux." It is not stated if HNO and NH_2OH can be reduced to NH_3 by this strain of *Bact. coli* in the presence of glucose.

It would seem that the reduction of NO_3 to NH_3 by bacteria is more general than supposed, and that the reduction must be seriously considered in assessing the importance of the oxidation of NH_3 to NO_3 by other micro-organisms in the general circulation of N in nature.

SUMMARY

1. Washed suspensions of *Cl. welchii* are able to catalyse the reductions of nitrate, nitrite and hydroxylamine to NH_3 by molecular H_2.

2. During the reduction of nitrate an appearance and final disappearance of nitrite can be demonstrated. This and other evidence makes it clear that nitrite is an intermediate in the reduction of nitrate.

3. There is some evidence (though not conclusive) that hydroxylamine may be an intermediate in the further reduction of nitrite.

4. Two strains of *Bact. coli* tested also brought about the reductions of nitrate, nitrite and hydroxylamine to NH_3 in the presence of molecular H_2.

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CCLX. OBSERVATIONS ON THE STABILITY OF XANTHINE OXIDASE

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IN the course of work on the isolation of xanthine oxidase from milk, much inconvenience arose from the instability of the preparations, which increased with each stage of purification. Rapid inactivation occurred when the bulk of the casein had been removed by precipitation with Na_2SO_4 and the diluted supernatant liquid was incubated at 38° for purposes of testing. The inactivation could be prevented by restoring the casein. This led to the hope that a non-protein stabilizer might be found, and, as described below, this hope has been fulfilled.

EXPERIMENTAL

All tests were carried out by the Thunberg technique using the following quantities: $0.1 M$ phosphate buffer, pH 7·0, 2·5 ml.; 1 in 5000 methylene blue, 0·5 ml.; $1.0 M$ glycine, 0·5 ml.; $0.0132 M$ xanthine, 0·2 ml.; the final volume was made up to 5·0 ml. Unless otherwise mentioned, the xanthine was added from the hollow stopper after temperature equilibration at 38°.

RESULTS

Table I shows that two-fold dilution of a whey preparation greatly affects its stability. Table II shows the restoration of stability by glycine. Table III shows that reactivation by glycine is also dependent on the time of incubation with glycine.

Table I. *The effect of dilution*

Reduction time

Incubation time in min.	0·5 ml. whey min. sec.		0·25 ml. whey min. sec.	
1	5	45	11	40
2	6	05	11	10
3	6	00	15	00
4	5	55	19	00
5	6	05	20	05
10	5	58	23	00
20	6	20	35	00

Table II. *Stabilization by glycine*

$1.0 M$ glycine ml.	Enzyme ml.	Incubation time in min.	Reduction time min. sec.	
—	0·25	2	7	0
—	0·25	10	120	0
0·5	0·25	10	5	30
0·5	0·25	60	7	55
1·0	0·25	60	6	50

The glycine was added to the tubes before incubation.

Table III. *Reactivation by glcyine*

Incubation time (min.) enzyme	Incubation time (min.) enzyme + glycine	Reduction time min. sec.	
0	2	5	30
10	0	16	00
10	10	10	15
10	20	7	50

The enzyme was first incubated anaerobically with buffer and methylene blue, then glycine was added and the tubes re-evacuated and incubated, and finally the xanthine was added from the stoppers.

Table IV. *Protection by glycine against copper*

1·0 M glycine ml.	Enzyme ml.	0·500125 M CuSO₄ ml.	Incubation time (min.)	Reduction time min. sec.	
—	0·25	—	2	11	10
0·5	0·25	—	2	5	30
—	0·25	0·05	2	150	0
0·5	0·25	0·05	2	5	45
0·5	0·25	0·05	23	5	30

The CuSO₄ was added to the other reagents in the tube before incubation.

As the distilled water then being used came from a copper still, it was suspected that the inactivation was due to copper poisoning [Wieland & Mitchell, 1931; Andersson, 1936], and that glycine was acting by forming a copper complex (Table IV). It was shown that glycine protected against the inactivation caused by added copper. Thenceforward all activity tests were done in presence of 0·1 M glycine.

In later work it was found that on dialysis at 0°, against large volumes of dilute buffer solutions, there was a loss of activity which could only be partially prevented by adding glycine (0·1 M) to the outer liquid and testing in presence of

Table V. *Activation and protection by KCN*

Batch of milk	Preparation	ml.	KCN (final conc.) M	Reduction time min. sec.	
46	Fat-free milk	1·0	—	2	25
			0·001	1	50
			0·03	1	30
			0·075	2	30
46	Decalcified milk	1·0	—	6	00
			0·015	2	45
			0·03	1	55
			0·045	1	55
			0·075	No reduction	
46	Decalcified milk 0·000125 M CuSO₄	1·0	—	No reduction	
			0·015	1	45
			0·03	2	05
			0·045	4	00
48	Fat-free milk	1·0	—	1	45
			0·015	0	45
			0·03	0	40
			0·045	0	48
49	(NH₄)₂SO₄ fraction	1·0	—	9	00
			0·0075	4	00
			0·015	3	15

Incubation time 2 min. in all cases.

a high concentration of glycine (1·0 M). However, on testing a dialysed preparation in presence of KCN, which has a greater affinity for copper than glycine, it was found that the activity could be not only restored, but actually increased above the original level. A number of different preparations were tested and all, including whole and fat-free milk, were found to be activated, the optimum KCN concentration lying between 0·01 and 0·03 M. At lower concentrations the activation is submaximal and at higher concentrations destruction of the enzyme occurs, as found by Dixon & Thurlow [1925]. KCN was also found to protect against inactivation by added copper. The KCN and xanthine were added together to the other reagents from the stopper in order to avoid the destruction produced by KCN alone [Dixon & Keilin, 1936]. As with glycine, the reactivation by KCN, after incubation at 38°, appears to be dependent on time. If tests are carried out in absence of glycine it is found that the reduction time depends on the length of the preliminary incubation. The interpretation of these results is, however, complicated by the fact that the enzyme cannot be incubated alone with KCN without being destroyed.

DISCUSSION

Wieland & Mitchell [1931] stated that KCN does not protect the enzyme against copper poisoning, but they did not test concentrations higher than 0·001 M. On the other hand, the high concentration required here suggests that the poison concerned is not silver, gold or mercury (cf. Wieland & Mitchell).

It appears from this that, during the process of purification, xanthine oxidase easily becomes inactivated by traces of copper, but it is not certain where the copper comes from. In the earlier stages of the work the distilled water contained traces, but since then a change has been made to a pyrex glass still. Boyden & Potter [1937] mention that their cellophane dialysis sacks contained copper. The cellophane used here does not contain enough to inactivate more than one batch of 50 ml. of enzyme preparation, and the same sack was used several times over, dialysis producing the same inactivation each time. Since there are traces of copper in milk [Davies, 1936], it seems more probable that the inactivation on dialysis is due to the removal of some substance which normally protects the enzyme against copper poisoning.

The inactivation of the enzyme does not appear to be due to the disappearance of —SH groups, for the following reasons.

(1) Milk has no detectable —SH groups.

(2) The concentration of KCN which activates milk instantaneously has only a very slow reducing action on the —SS— groups in milk.

(3) Addition of cysteine, when testing the activity of milk, causes no activation.

From the practical point of view, if accurate activity measurements are required, it is recommended to use both glycine (final conc. 0·1 M) and KCN (final conc. 0·01–0·03 M). Since KCN can only be added together with the xanthine, after temperature equilibration is complete, it is advisable to retain glycine to avoid the inactivation which may occur during that time.

SUMMARY

1. Glycine and cyanide can increase the activity of xanthine oxidase in whole milk and concentrates.

2. This is probably due to removal of copper inhibition.

3. The loss of activity of some preparations on dialysis or incubation can be wholly or partly reversed in the above way.

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CCLXI. INVESTIGATIONS INTO THE METHOD OF ESTIMATING VITAMIN E

III. THE RELATION BETWEEN DOSAGE AND RESPONSE TO VITAMIN E

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IN previous communications [Bacharach *et al.* 1937; Bacharach & Allchorne, 1938] we proposed the use of virgin rats as test animals for vitamin E. We also [Bacharach & Allchorne, 1938] defined the sense in which we use the terms "positive response", "live litter" and "fertility rate" or "percentage fertility".

It must be emphasized that the biological assay of any vitamin cannot be carried out until

(1) A stable preparation of the vitamin is available in adequate amounts for use as a standard.

(2) A unit has been defined in terms of this standard.

(3) Information is available as to the relation between response to the vitamin and dosage.

For vitamin E, condition 1, and therefore condition 2, is not at present fulfilled. The need for an agreed standard and a defined unit may of course be circumvented if the pure vitamin, such as one of the tocopherols, becomes available instead. Assays could then be expressed as vitamin E activity in terms of an equivalent weight of tocopherol, but this also could seldom be done with even approximate accuracy unless condition 3 were satisfied.

Having these considerations in mind, we turned our attention about two years ago to establishing the relation between response to vitamin E and dosage, with a view to constructing an average response curve. Such a curve would prove of use for assay purposes when conditions 1 and 2 are satisfied, but it could meanwhile be of service in permitting relative vitamin E activities to be assigned to two materials.

In any endeavour to construct a response curve involving the use of large numbers of animals, a vicious circle is likely to be established. The test must be extended over a considerable period of time, but, in the absence of a quantitative method of test and an agreed standard, there is no convincing means of demonstrating that the test substance has itself undergone no change during the test period.

Precise significance must be given to the word "dosage" in all vitamin tests. For reasons that will be mentioned below we make use of the total dose administered to each animal, that is, the product of the daily dose and the number of days of administration, when the dose is uniformly divided over that number of days.

The practical importance of some of the considerations discussed above was brought forcibly home to us during our first attempt to establish a dosage-response curve, and again later. A large sample of freshly extracted wheat-germ oil was placed in a stoppered bottle and kept in the refrigerator. Vitamin E-deficient female rats were given doses of this oil at several levels, the numbers

of animals that had been submitted to each dose by any given date being kept approximately equal. Examination of the results after many weeks' experiment pointed to a falling activity of the oil, or to an altered sensitivity of the animals, or both (Table I). The former seemed especially probable as the oil was found after 9 months of use to have developed a peroxide value of 43; this had risen to 58 some 10 weeks later. It seemed to us almost certain that the oil must also have undergone loss of vitamin E during the period of test. This made it impossible to do as originally planned, namely, to compare the vitamin E thresholds of virgin and of R.P. animals, a matter that is also referred to in our earlier publication [Bacharach & Allchorne, 1938]. Examination of our figures showed irregular fluctuations in the responses both of R.P. and of virgin animals, and the experiment was abandoned. A similar attempt to construct a curve for response of R.P. animals to cotton-seed oil was abandoned for similar reasons. A dose giving 33 % fertility with R.P. animals gave 100 % and 38 % with 11 and 8 virgin animals respectively. At other levels virgin animals showed responses roughly graded to dosage, thus

Dose (mg.)	No. of animals	Fertility %
562·5	25	4
1125	16	19
1687·5	19	74

This experiment was carried on for about 6 months. Comparison of the above figures with those shown for virgin animals in Table I illustrates the difficulties of evaluating quantitatively one substance in terms of the other.

Table I. *Effect of wheat-germ oil on fertility rates*

(No. of animals in brackets)

			R.P. animals		
			Groups in rough chronological order		
Dose mg. (total)	Virgin animals	Total	1st	2nd	3rd
112·5	4·5 (22)	0 (16)	0 (10)	0 (6)	—
562·5	0 (8)	10 (21)	20 (10)	0 (10)	0 (1)
1125·0	17 (12)	25 (33)	50 (10)	10 (10)	10 (10)
1687·5	57 (7)	68 (31)	80 (10)	90 (10)	40 (10)

We decided next to avoid some of the pitfalls inherent in the use of vegetable oils subject to peroxidation, by making use of a concentrate whose satisfactory biological activity had first been verified in a short exploratory test. This sample, from which most of the sterols had been removed by simple freezing, consisted of unsaponifiable matter from freshly prepared wheat-germ oil; it was sealed off under N_2 in quantities of between 3 and 5 g. and kept in the refrigerator. Fresh tubes were opened as they were wanted and the contents of only one tube were in use at any one time. Further, attempts were again made to keep the numbers of animals at the various levels of dose as nearly equal as possible up to any given time, in order that any slight fall in vitamin E dosage might affect animals on the different doses to roughly the same extent.

The results of this work are shown in Table II and in Fig. 1, which we believe to be the first published curve showing the relation between dosage and response to vitamin E. In this curve the dose has been expressed in arbitrary units; actually, one "unit" corresponded with a total dose of 5·625 mg. of the wheat-germ oil concentrate.

Table II. *Relation between total dosage of wheat-germ oil concentrate and fertility rate*

Dose mg. (total)	Animals		Fertility rate (%)
	Implanted	Fertile	
—	460	0	0
5·625	46	7	15·2
11·25	64	20	31·3
22·50	40	35	87·5
45·00	31	29	93·6
90·00	31	30	96·8
180·00	20	20	100

Some of the animals in this test received their total dose divided uniformly over a period of 5 days and others over a period of 10 days. During the earlier part of this test it appeared to us that the longer dosage period was proving more effective than the shorter. However, as time went on and the increasing numbers of animals at each level gave to the respective fertility rates an increasing significance, we were unable clearly to establish this difference; for the purpose of constructing the curve and Table both periods of dosage have been included, as well as a period of 8 days for some of the animals receiving 90 mg. of extract (16 "units").

We still consider it possible that the method of distributing doses with time may have some effect on response, but it seems clear that only the use of a much larger number of animals could demonstrate a significant difference between, for example, the division of the total dose over 5 days or over 10 days. It is certainly desirable therefore, in any comparative tests, to make use of a fixed dosage period; we ourselves have standardized this at 10 days. The effect of dosage period on response has, of course, been recorded for other vitamins [cf. Bacharach *et al.* 1936].

The curve shows certain features that call for comment. Theoretically, since it illustrates a typical instance of so-called "quantal" response, it should be sigmoid.

Fig. 1. Dosage-response curve for vitamin E. This curve is constructed from the equation to the regression line, calculated as described. × =fertility found by experiment. Figures in brackets denote nos. of animals. 20 animals receiving a dose with ratio 32 gave 100% fertility, the calculated value being 99·9%. This value was not used in calculating the equation to the regression line, and is not shown in the figure.

It is due to the relatively small number of animals used that this sigmoid structure is not revealed by the lower doses and fertility rates. If, for example, a dose of 1 mg. or 2 mg. produces an extremely low response, about 1 or 2%, this would be expressed in an almost horizontal part of the curve near the origin, but the dose would have to be administered to 100 animals or more for such values to be established. When the curve relating % fertility to dosage ratio is constructed by appropriate transformation of the equation to the regression line, discussed below, the sigmoid portion near the origin becomes evident (see Fig. 1).

Assuming therefore that the relation between dose and fertility is similar to that found for other types of quantal response, the relation between the logarithm of the dose and the normal equivalent deviation of the response, or the "probit" of Bliss [cf. Irwin, 1937], should be linear. A regression line can be calculated from these values by the usual method of least squares. Such a regression line, or the equation to it, can be used not only for comparing the vitamin E contents of two substances, but also for calculating the error of the comparison.

With the co-operation of Dr J. O. Irwin, the figures given in Table II have been used for calculating the equation to the regression line, based on responses to the doses 1, 2, 4, 8 and 16, and it is found to be

$$y = 0.87x + 3.87,$$

where y is the probit of the response and x is the logarithm (to base 2) of the ratio of the dose to 5.625 mg. By further refinement it is possible to take into account the group of animals that showed a 100 % response to the dose ratio of 32. The derived equation is for a line with a slightly steeper slope. The difference between the results obtained from the corrected and the uncorrected equations lies so far within the range of experimental error inherent in this type of test that the extra labour involved in calculating the corrected equation is not justified.

From the equation the particular concentrate used is found to have a mean fertility dose of 2.5 "units" (13.8 mg.), with probable limits of error, for P=0.99, 71 % to 141 %, and, for P=0.95, 77 % to 129 %. Further, it has been calculated that the relation between probits and logarithms of doses shows no significant departure from linearity.

The equation to the regression line makes it possible, albeit with an accuracy inversely related to the square root of the number of animals used, to find with a single group the mean fertility dose of a source of vitamin E; the more the found fertility departs from a fertility rate of 50 %, the greater the error of the test. By a simple transformation of the equation to the regression line, it can be shown that

$$\log D_M = \log D_T - 0.35 y_T + 1.74,$$

where D_M and D_T are the mean fertility dose and the test dose respectively, and y_T is the probit of the response given to the dose D_T. In the example illustrated in Table IV and mentioned again below, the two values for the mean fertility dose obtained from the groups of animals given the two doses of wheat-germ oil are calculated to be 300 and 210 mg., with a weighted mean of 260 mg. The weighted mean value obtained by graphical methods from the standard response curve was 280 mg. The direct dosage-response curve is very steep, and for most purposes it will be found sufficient to use it rather than the derived regression line, as the above example shows, provided that the responses involved fall on the approximately straight part of the curve.

Certain practical considerations emerge from the exceptional steepness of the curve. A 300 % increase of dose, from 1 to 4 "units", has brought about a nearly 500 % increase in the fertility rate, from 13 % to over 70 % fertility. When larger numbers of animals are taken into account, experimentally determined points should lie still closer to the plotted curve, but it seems unlikely that its general shape would be materially changed, and one must therefore anticipate that testing vitamin E by the fertility rate method at levels producing below 25 % or above 75 % fertility is liable to show a high degree of error. On the other hand, the steepness of the curve has a compensating advantage;

provided that a fertility rate near to 50 % results from the administration of a particular dose, the equality of that dose with a dose of other material giving the same fertility rate can be asserted with some confidence. The steepness of the curve, in other words, means that the range over which tests can be accurately carried out is restricted, but that within this range, the accuracy is considerably higher than might have been anticipated for results obtained with small numbers of animals.

A response curve of the type shown affords certain useful information. For example, it may be required to know how much vitamin E is to be added to some synthetic diet in order to make it an adequate source for full fertility. Inspection of the curve shows that twice the mean fertility dose is insufficient, that three times that dose is nearly adequate, and that six times is almost certainly a safe addition for the purpose.

Since, however, the curve is based on the behaviour of completely deficient animals, it is clear that much smaller amounts would be required daily if they were administered throughout the lives of the animals. If M be the mean fertility dose found for a substance administered over 10 days to virgin animals about 100 days old, presumably a daily dose of $6M/100$ would be quite adequate if given daily from birth, or even from weaning. A daily dose of $M/10$ would allow a margin for any inefficient storage of the vitamin during infancy and adolescence. A sample of wheat-germ oil whose M.F.D. is found to be 1 g. should confer full fertility if fed from weaning in daily doses of 100 mg. to animals on a diet otherwise completely free from vitamin E, always assuming that the oil undergoes no serious deterioration during its period of administration. We have records of 87 % fertility (15 animals) and 84 % fertility (6 animals) for two different samples of wheat-germ oil given in daily prophylactic doses of 112 mg.

Table III. *Vitamin E activities of α- and β-tocopherol*

Substance	Dose mg.	No. of animals	Fertility rate (%)
α-Tocopherol	1·125	5	40
	2·25	6	50
	4·50	5	100
β-Tocopherol	0·72	6	0
	1·8	1	0
	2·25	9	0
	2·70	1	100
	3·6	6	83

It may be of interest to give a few examples of the use of the response curve in comparative tests. We have published elsewhere [Bacharach, 1938, 2] figures for a test carried out with highly purified samples of the allophanates of α- and β-tocopherol. Material was available for administration to a few more animals after we had made that communication, and the consequently revised comparison is shown in Table III. The M.F.D. of α-tocopherol is considered from the results shown to lie in the neighbourhood of 1·5 mg. (0·8 × 1·9 mg.; the factor 0·8 represents conversion of the allophanate into the free alcohol). The activity of β-tocopherol appears to be rather over half that of α-tocopherol, but the error of this test is extremely high. Indeed, it is doubtful whether these or any other published figures finally establish a difference in biological activity of the two forms of tocopherol. Their activities call for some further comment. Concentrates used for isolation of the crystalline allophanates generally exhibit a M.F.D. of something between 15 and 30 mg. At worst, then, they should

contain 5 % of α-tocopherol or rather under 10 % of the β form; at best they should contain twice these quantities. Yet yields of even the crude mixed allophanates have never been reported as much above 1 % of such concentrates, suggesting that the process of isolation may result in loss or destruction of up to 90 % of the active substances [cf. Bacharach, 1938, 1].

Table IV. *Vitamin E activities of wheat-germ oils and concentrates*

	Dose mg.	Fertility rate (%)	M.F.D. from curve (mg.)	No. of animals	Weighted M.F.D. (mg.)
Wheat-germ oil A	255	43	270	7	} 280
	450	83	310	6	
Wheat-germ oil B	450	0	>1100	7	} 700
	900	84	600	6	
Wheat-germ oil C	450	75	320	4	} 250
	675	100	<250	6	
Concentrate from C	20	57	18	7	} 15
	40	100	<15	7	

A comparison of two samples of wheat-germ oil is given in Table IV. From the figures it is concluded that one oil is about three times richer than the other in vitamin E. Here again the error is very large; it is quite possible that the activities of the oils do not differ by more than 50 %. Again, a sample of wheat-germ oil concentrate was compared with the oil from which it was made. It seems that an approximately 16-fold concentration has been achieved.

Whether or not the relation between dosage and response found under our conditions of test would also apply generally is by no means to be taken for granted. Nevertheless, we believe our procedure to be of general application and to show clearly that comparisons of vitamin E activity should always be made by establishing, under as nearly identical conditions as possible, the mean fertility doses of the materials in question.

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ADDENDUM

We have had the privilege of reading in proof the recent paper by Mason & Bryan (*Biochem. J.* 1938, **32**, 1785), wherein they point out that we have omitted in our earlier publications to state whether or no our experimental animals (that is, the young does ultimately to be used in the test) themselves

had access to their mothers' diet before they were weaned and, if so, what that diet consisted of. We would seize this opportunity to rectify the omission. No steps are taken to prevent the sucklings from eating their mothers' diet, which is replaced by our standard vitamin E-free diet either the day after weaning or, if on that day they weigh under 35 g., when they have reached this weight, which is at most 2 days later. The stock diet that these young does may therefore be wholly or partly consuming for 10 to 15 days, preceding their transference to the vitamin E-free diet, is practically identical with that described in an earlier publication (Bacharach, *Biochem. J.* 1933, **27**, 5). It consists of wholemeal flour, full-cream dried milk and yeast extract, supplemented with limited amounts of green leaves, liver (or lean meat) and fish: the supplements, however, are withdrawn towards the end of their mother's pregnancy and are not restored until after the litter has been weaned.

CCLXII. THE EFFECT OF DIFFERENT METHODS OF DRYING ON THE BIOLOGICAL VALUE AND DIGESTIBILITY OF THE PROTEINS AND ON THE CAROTENE CONTENT OF GRASS

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ONE of the outstanding features claimed for modern methods of artificial grass drying is that they involve but little destruction of the nutritive properties of the grass. The high temperatures attained during the process, however, raise the question of heat injury to the proteins. It is well known that heating lowers the biological value of the nitrogen of various foodstuffs [Morgan, 1931; Kon & Markuze, 1931; Fixsen & Jackson, 1932; Maynard & Tunison, 1932; Morgan & Kern, 1934; Morris *et al.* 1936]. Morris *et al.* [1936] found, on the whole, little difference between the biological values for cows of the proteins of fresh and artificially dried grass, though with spring grass there was an indication that the dried product was inferior. In view of the increasing use of artificially dried grass in the feeding of pigs and of poultry and of marked differences between herbivora and omnivora in the utilization of the nitrogen of feeds it was of interest to study on another animal the effect of heat treatment on the proteins of grass. Rats have been extensively used for such tests. They respond readily to even small alterations in the chemical make-up of the nitrogenous constituents of their diets. Further, it is probable that they are less able than ruminants to utilize breakdown products of proteins and are therefore more sensitive in indicating the possible detrimental effects of heating. It is probably safe to assume that if a rat test discloses no difference between two products, a feeding test on cows would likewise show no difference. On the other hand, a definite difference in this respect found with rats would certainly call for a further investigation with ruminants.

For these reasons it was decided to carry out nitrogen balance experiments on rats. The carotene content of the grasses dried under various conditions was also studied, as well as the rate of destruction of carotene in the process of natural curing.

EXPERIMENTAL

(1) *The drying and preparation of grass*

Compared with that of herbivora, the capacity of the intestinal tract of the rat is limited and it was necessary in these experiments to cut down as far as possible the bulk of the diet by selecting a grass of very high protein content. For the same reason (apart from other difficulties) it was not possible to feed the grass fresh and only dry samples could be compared.

(2024)

Grass was taken from permanent grassland with a first class sward about 4–6 in. in length. The chief plants in the herbage were wild white clover and perennial rye grass. The mower was run over the meadow on 25 June 1937 to remove all long grass and on 21 July the grass was cut with a close cutting mower with an elevator attached so that the freshly cut grass was loaded on a cart immediately and taken to the farm buildings for drying.

The details of the various treatments applied to this batch of grass were as follows.

(a) Grass dried artificially in a grass drier at the normal running temperature (300° F.).

The drier used was a Kaloroil drier of the rotating drum type heated by oil fuel.[1] A full description of this type of drier will be found in the report by Roberts [1937]. The inlet gases were heated to about 300° F. (variation 275–325° F.) but the outlet temperature could not be recorded.

The grass was removed from the drier as soon as it was sufficiently dry (15–30 min.) and it is probable that only the outside of the grass ever reached a temperature approaching 300° F. The freshly dried grass was crisp but after removal from the drier quickly became less powdery. The interval between cutting and drying this sample was 6 hr.—i.e. 2 hr. on the cart and 4 hr. exposure on a wooden platform under a Dutch barn. The condition of this sample after drying was excellent and it was dark green in colour. It was stored in sacks for 15 days and was then made into bales under high pressure and was covered with sacking.

(b) Grass dried artificially in a grass drier at low temperature (170° F.).

The interval between cutting and drying this sample was 6½ hr. It was dried in the same plant as the first sample but the temperature of the inlet gases was kept at about 170° F. (variation 150–200° F.). The time required for drying was much longer and it was necessary to keep the grass in the drum for about an hour (50–70 min.). A well-dried dark green sample of grass was eventually obtained and the after treatment was the same as for the first sample.

(c) Grass dried artificially in a current of hot air.

A third batch of the grass was placed half an hour after cutting in a tunnel-shaped drying oven through which passed a current of air heated by a hot water radiator and propelled by a fan. The temperature of the inlet air was about 135° F. (variation 104–142° F.). The time of drying was 22 hr. This sample was sacked but not pressure-baled.

(d) Grass sun-dried under natural conditions (hay).

A further batch of grass was unloaded from the cart 2 hr. after cutting. It was intended to place the grass in the open at once but as heavy rain fell while unloading it was left overnight indoors spread on a floor. The depth of the layer was about 8 in. and it was turned to avoid undue fermentation. Twenty-one hr. after cutting the grass was spread in the open in a layer about 3 in. thick. The weather to which the grass was subjected was unsettled and dull, and heavy rain fell in the 36th hr. after cutting. The grass was turned on the 3rd day. In order to complete the drying on the 5th day after cutting the grass was turned three times and finally placed in a small stack and thatched with straw. The hay had lost most of its green colour and parts were slightly blackened but would be described by a farmer as moderate quality hay. Ten days later this hay was pressure-baled and the bales were covered with sacking for storage.

In addition to these samples, a further batch of grass was cut from the same meadow one day later and was treated as follows.

(e) *Grass dried in air at normal temperature without sun or rain.*

The grass was cut, carted and off-loaded after an hour. It was spread about 3 in. deep on a concrete floor in a room with screened windows. The grass was turned twice daily to prevent fermentation and was thoroughly air-dried in 12 days. It was pressure-baled 14 days after cutting and the bales were covered up with sacking. The hay lost much of its green colour during curing but otherwise appeared to be of very good quality.

Finally, to study the rate of destruction of carotene in the course of curing, part of a different meadow was scythed between 11 and 11.30 a.m. at the beginning of September, the grass was left in the field in a layer of about 3 in. and samples were withdrawn at intervals for carotene estimations. This field had been previously cut at the beginning of June. Between then and the second cutting the weather was, on the whole, very dry, and the grass made little growth; it was 6–7 in. high when cut and rather parched. During curing the weather was, on the whole, bright and sunny with no rain.

(2) *Determination of the biological value and digestibility of the proteins of dried grass*

It was technically not possible to compare simultaneously more than three types of dried grass and the tests were therefore limited to three products of practical importance: two samples of grass dried in the drier at high and low temperature and the sample of hay. The feeding experiments started $5\frac{1}{2}$ months after the grass had been dried.

The grasses dried at high and low temperature contained respectively 23·5 % and 23·1 % of protein (N × 6·25) on the dry basis. The sun-cured sample contained more, 25·1 %, probably owing to relatively greater loss of fermentable non-nitrogenous constituents.

The grasses were milled to a fine powder and were mixed in suitable proportions with a "nitrogen-free" basal diet to yield mixtures containing about 9 % protein (N × 6·25) on the dry basis. The "nitrogen-free" diet had the following composition:

Butter fat	17
Rice starch	51
Potato starch	10
Sugar	17
Salts (Steenbock 40)	5

The biological value and true digestibility of the grasses were measured by the method of Mitchell [Mitchell, 1924; Mitchell & Carman, 1926] as described in detail by Henry *et al.* [1937]. Because of the nature of the grass diets, it was necessary to mix them for feeding with a relatively large quantity of water (more than $1\frac{1}{2}$ times the weight of the diet). Twelve rats were used and on each the three types of grass were tested in turn. The results are given in Tables I, II and III. Table I gives the individual nitrogen metabolism data, Table II gives average values and Table III contains a statistical examination of differences in biological values. Figures for true digestibilities were so similar (Table II) that statistical tests were considered superfluous. The statistical method used was that of the paired *t* test of "Student" [1908; 1925] as described by White [1937]. When comparing two substances results obtained on the same rat in different periods were paired. The results show quite conclusively that sun-drying was more detrimental than artificial drying at either high or low temperature. The figures also indicate that the proteins of grass dried at low temperature were inferior to those of the high-temperature grass but the evidence to this

Table I. *Nitrogen metabolism; individual data for 6-day periods*

Rat no.	Type of grass*	Initial wt. g.	Final wt. g.	Diet intake† g.	N intake mg.	Faecal N mg.	N in urine mg.	Biological value	True digesti-bility
				Low egg-nitrogen diet					
1	—	64	70	46·11	—	101·5	100·0	—	—
2	—	65	69	46·94	--	89·8	102·2	—	—
3	—	69	73	44·91	—	92·9	110·9	—	—
4	—	59	64	37·67	—	93·6	98·2	—	—
5	—	58	60	31·82	—	73·5	111·1	—	—
6	—	60	68	49·59	—	105·0	113·8	—	—
7	—	64	71	43·73	—	103·8	106·8	—	—
8	—	62	66	37·38	—	75·6	101·2	—	—
9	—	64	73	47·45	--	97·8	106·2	—	—
10	—	60	63	40·61	—	99·4	108·3	—	—
11	—	59	62	35·14	—	90·4	98·4	—	—
12	—	61	62	38·86	—	98·0	106·3	—	—
				1st grass period					
1	S	68	69	40·31	566·4	265·7	297·5	51·7	69·9
2	H	71	72	38·59	544·9	275·1	257·9	57·3	64·5
3	L	72	75	44·54	624·5	311·8	274·6	59·7	64·9
4	S	61	59	34·93	490·8	257·9	282·2	39·5	64·3
5	H	64	66	40·32	569·3	302·6	241·1	66·0	63·9
6	L	64	64	35·54	498·3	261·6	264·9	50·2	63·1
7	S	71	67	36·70	515·6	264·8	269·8	50·8	65·7
8	H	70	72	42·78	604·1	308·6	273·8	57·2	63·5
9	L	71	73	41·17	577·2	282·8	288·3	53·1	66·5
10	S	63	63	42·04	590·7	297·0	328·1	43·5	67·1
11	H	62	68	47·51	670·8	331·0	261·5	65·9	69·2
12	L	62	66	44·62	625·6	312·6	294·6	55·8	68·5
				2nd grass period					
1	H	77	79	45·39	640·9	373·2	225·7	73·4	59·6
2	L	77	82	48·14	674·9	364·2	255·3	67·4	62·5
3	S	79	80	47·49	667·2	376·1	275·1	59·1	58·5
4	H	64	69	42·34	597·8	299·3	228·0	66·9	65·7
5	L	70	73	45·07	631·9	322·5	246·3	71·0	66·9
6	S	68	70	43·75	614·7	336·9	284·1	54·0	61·3
7	H	72	83	55·70	786·5	381·6	262·1	72·0	68·6
8	L	73	77	46·52	652·2	348·4	272·5	60·3	61·6
9	S	75	74	44·41	623·9	333·6	316·5	47·4	62·8
10	H	68	71	45·58	643·6	261·5	245·8	72·3	76·5
11	L	71	73	45·86	643·0	344·2	239·9	69·7	66·1
12	S	66	68	44·30	622·4	333·7	299·8	52·0	65·3
				3rd grass period					
1	L	78	81	53·34	747·8	411·7	347·4	54·8	64·0
2	S	79	83	51·56	724·4	370·4	319·7	58·1	66·8
3	H	84	94	57·96	818·4	444·6	257·0	73·0	60·6
4	L	71	78	49·42	692·9	339·5	263·3	65·4	66·0
5	S	76	78	49·93	701·5	355·4	314·6	60·4	68·0
6	H	73	83	57·23	808·1	430·7	269·0	70·6	63·2
7	L	85	91	56·00	785·1	423·8	276·6	68·2	63·5
8	S	75	81	53·82	756·2	404·2	331·3	53·7	61·8
9	H	75	85	57·14	806·8	426·5	294·5	65·3	64·1
10	L	76	84	56·31	789·5	382·1	278·8	70·3	68·8
11	S	74	76	50·86	714·6	418·9	309·0	52·1	61·6
12	H	71	79	52·10	735·7	366·5	281·7	66·7	69·5
				Low egg-nitrogen diet					
1	—	80	84	38·57	—	108·9	138·6	—	—
2	—	85	86	34·18	—	92·8	122·0	—	—
3	—	96	101	48·98	—	103·9	130·8	—	—
4	—	83	95	55·09	—	108·6	119·6	—	—
5	—	78	80	34·53	—	94·9	122·6	—	—
6	—	87	96	57·36	—	137·7	132·4	—	—
7	—	95	105	59·13	—	146·6	126·6	—	—
8	—	85	91	41·92	—	91·6	126·4	—	—
9	—	92	93	43·32	—	108·5	130·7	—	—
10	—	87	95	52·81	—	126·6	125·0	—	—
11	—	77	81	40·19	—	118·0	116·2	—	—
12	—	85	85	38·41	—	107·2	120·0	—	—

* S = sun-dried. H = artificially dried at high temperature. L = artificially dried at low temperature.

† On the dry basis.

Table II. *Biological values and true digestibilities of three types of dried grass*

| | Sun-dried | | Artificially dried | | | |
| | | | High temperature | | Low temperature | |
Period	Biological value	True digestibility	Biological value	True digestibility	Biological value	True digestibility
I	46·38	66·75	61·60	65·28	54·70	65·75
II	53·11	61·97	71·15	67·60	67·10	64·28
III	56·08	64·55	68·90	64·35	64·68	65·57
Mean	51·86	64·42	67·22	65·74	62·16	65·20

Table III. *Statistical significance of the mean differences in the biological values of the three types of dried grass*

Difference	Standard error of mean	P*
High temperature – Sun-dried	+ 15·36 ± 2·63	1 : 5000 Significant
Low temperature – Sun-dried	+ 10·30 ± 2·81	1 : 263 Significant
High temperature – Low temperature	+ 5·06 ± 2·85	1 : 10 Not significant

* P = probability that a mean difference at least as great as the observed mean difference would have arisen by random sampling from a homogeneous population.

effect is inconclusive (Table III). The lowering of the biological value of the proteins in the sun-curing of grass is most probably associated with the fermentative changes taking place during the process. It is sufficiently marked, as judged on rats, to invite an investigation on ruminants. According to Saakian [1935] artificially dried hay was 20 % higher in feeding value than ordinary hay from the same meadows.

Nevens [1921] has measured on rats the biological value and true digestibility of the proteins of alfalfa (lucerne) hay and obtained values of 62 and 58 respectively. The former figure is definitely higher than that obtained in the present work for sun-cured grass, slightly lower than that for grass dried at high temperature and equal to that for the low-temperature grass. The digestibility coefficient of the lucerne hay was lower than that of the artificially dried grasses which amounted to about 65.

(3) *The effect of drying on the carotene content of the various grasses*

The carotene was estimated by the method of Ferguson & Bishop [1936]. The results will be found in Tables IV and V of which the first gives the carotene

Table IV. *The carotene content of grass dried by various methods*

| Method of drying | % moisture | Mg. carotene/100 g. grass | | Carotene content as % of that present in fresh grass |
		Moist	Dry	
Fresh grass	88·92	10·4 10·2	49·3 48·5 } 48·9	100
Artificially dried high temperature	10·56	39·1	42·4	86·7
Artificially dried low temperature	12·90	32·1	38·2	78·1
Artificially dried in current of hot air	7·89	39·0	43·7	89·4
Sun-dried under natural conditions (hay)	21·38	9·6	12·2	25·0
Dried without sun at air temperature	18·06	12·6	15·4	31·5

Table V. *Rate of destruction of carotene in the sun-curing of grass*

Hours after cutting	Time of exposure to sunshine* hr. min.	% Moisture	Mg. carotene/100 g. grass		Carotene content as % of that present in fresh grass
			Moist	Dry	
Freshly cut	—	65·27	8·7	25·0	100
1	54	59·05	9·0	22·0	88
3	2 54	47·78	11·0	21·0	84
6	5 27	38·59	10·3	16·8	67·2
24	9 57	35·54	9·9	15·4	61·6
48	16 32	35·09	9·1	13·4	53·6
72	24 32	24·78	4·7	6·3	25·2
96	30 45	16·17	4·4	5·3	21·2

* As measured by a Jordan photographic sunshine recorder.

content of the grasses used in the protein experiment and the second shows the changes in the carotene content of grass in the course of its conversion into hay.

In both cases hay cured under natural conditions contained only about one quarter of the carotene originally present in the fresh grass. Artificial drying at normal running temperature preserved nearly 90 % of the carotene. This figure is in good agreement with the earlier findings of Watson *et al.* [1933]. The drying at the lower temperature was slightly more injurious, most probably because of the longer exposure of the material to the hot gases after it had become relatively dry [Watson, 1934] and it is possible that the same reason would account for the slight lowering of the biological value of the proteins described on p. 2028.

The high figure for the carotene content of the sample of grass dried in a hot air tunnel is rather surprising as the drying took some 24 hr. and air was continuously passing over the grass; on the other hand the temperature of the air never rose above 142° F. and that of the grass must have been even lower for, at any rate, part of the time.

The measurements of the rate of disappearance of carotene when grass is made into hay show a fairly steady fall. The loss after 48 hr. is some 46 % of the original value. This compares unfavourably with the figures given by Greenhill [1936], but it must be remembered that his results were obtained in the course of partial field drying of grass as a preliminary to artificial drying and not in haymaking. On the other hand, the figures agree quite well with the data of Dexter & Moore [1937] for lucerne hay cured in swaths.

SUMMARY

1. Grass was dried by artificial means and also made into hay in the normal way and the effects of the various procedures on the carotene content and on the biological value of the proteins of grass were measured.

2. The biological value and true digestibility of the proteins of grass artificially dried at the normal running temperature (300° F.) in a rotating drum type drier were compared on rats by the method of Mitchell with those of grass dried in the same drier at low temperature (170° F.) and those of sun-cured hay. All three samples were made from one bulk of fresh grass.

3. The following figures were found for biological value and true digestibility: artificially dried grass, high temperature, 67 and 66; artificially dried grass, low temperature, 62 and 65; hay, 52 and 64.

The differences in biological values of the proteins between artificially dried samples and hay were statistically highly significant. Other differences were not.

4. Carotene figures are given for various samples of dried and fresh grass and also measurements of the rate of loss of carotene in the course of sun-curing of grass.

Our thanks are due to Prof. H. D. Kay and Mr J. Mackintosh for advice during the course of these experiments; also to Mr J. E. Campion who assisted in operating the grass-drying plant.

Note added 28 October 1938. Smuts & Malan [1938] have obtained very recently for lucerne meal biological values of 60 and 61 which agree very well with the findings of Nevens [1921].

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CCLXIII. THE CATATORULIN TEST FOR VITAMIN B$_1$

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THE catatorulin test, using avitaminous pigeon's brain, may still occasionally prove useful, because it is fairly sensitive, reacting to less than $0 \cdot 2\gamma$ vitamin B$_1$, and because it has a low sensitivity to the phosphoric esters [Peters, 1937]. With the newest thiochrome test [Jansen & Westenbrink, 1938] small amounts of vitamin B$_1$ are evidently determined, but the Schopfer mould test does not distinguish between the different forms of vitamin [Sinclair, 1937], and the new yeast enzyme test does not separate the free vitamin from its monophosphoric ester. Further there are points of general application in the use of a tissue preparation of this nature for an enzyme test in which it is found that fine grinding or extraction of the tissue leads to loss of activity [McGowan & Peters, 1937]. For these reasons the collective experience of some years with this test is here recorded briefly; in particular, it is believed that a satisfactory theoretical basis has been found for the use of pyruvate and that the conditions have now been defined which make for the maximum degree of sensitivity.

The first catatorulin tests for vitamin B$_1$ using avitaminous pigeon's brain were based upon the increase in O$_2$ uptake observed in lactate solution as the result of adding the vitamin [Passmore *et al.* 1933]. This was extended to lactate and pyrophosphate [Kinnersley *et al.* 1935], when it was found that this gave larger differences. Finally it became clear that pyruvate rather than lactate was the essential substrate; attention was therefore diverted to pyruvate [Ogston & Peters, 1936], especially in view of the further increase in the O$_2$ differences which was thus obtained. At the same time these last changes meant giving up an equation which had served well previously with lactate for the 2nd hour of respiration. Recent papers from this laboratory [Peters, 1936; McGowan & Peters, 1937] show clearly the incorrectness of the theoretical basis of this Michaelis equation in which there was postulated a reversible equilibrium between vitamin B$_1$ and some essential component of the system. Now we know that a quite different series of reactions is involved. In the absence of vitamin B$_1$, some component (probably protein) of the pyruvate-oxidase system becomes unstable; this is proved by the fact that the effect of vitamin B$_1$ tends to be that of maintenance [Peters *et al.* 1935; Westenbrink & Polak, 1937]; the control decreases in respiration rate more rapidly than the sample containing vitamin, and also the washed avitaminous brain tissue gives a poor catatorulin effect which is not improved by the addition of the washings. The steadier values obtained for respiration during the second hour are only in part due to the removal of the residual substrates; they are probably associated with the inactivation of the unstable component of the system; the rate of inactivation increases with rising temperature. In lactate the series of changes summarized by the previous equation is complicated:[1] (*a*) lactate is converted into pyruvate; (*b*) vitamin B$_1$

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[1] Another possible explanation of the catatorulin effect might be that added aneurin merely prevents the dissociation of the last traces of pyrophosphoric ester, but this is not consistent with the trifling effect of vitamin upon the normal brain.

reaches its unstable component by diffusion; (c) the vitamin may first have to be phosphorylated, a question which is not yet decided [Peters, 1937; Ochoa & Peters, 1938]. By using pyruvate (a) is eliminated.

In arriving at a new theoretical treatment for pyruvate as substrate it must be remembered that the Q_{O_2} observed has three components, (1) residual respiration (not due to pyruvate), (2) residual pyruvate respiration (due to residual traces of vitamin B_1) and (3) catatorulin respiration, due to added vitamin. We may call $(1) + (2)$ R. If we assume that the added aneurin a acts either as a prosthetic group or coenzyme to some protein, p, we can write ap as the actual enzyme system. At any time the catatorulin O_2 uptake in the absence of complicating factors depends upon ap. Since p is very unstable at 38° in absence of aneurin and substrate [Peters, 1936], at any time after 30 min. no more ap can be formed; after this time therefore

$$Q_{O_2} \text{ observed} = k \ (R + ap).$$

We should expect the difference between the Q_{O_2} with and without added vitamin to remain constant after the 30 min. period, if the complete system is reasonably stable, because the diminution of the residual respiration (1) will be the same for each, and the residual traces of the pyruvate-oxidase system will remain equally stable. The curves shown in Fig. 1 are consistent with this view, which has been previously expressed.

For the period of respiration $\frac{1}{2}$–2 hr. there is a fairly constant change produced by the aneurin in $\mu l./g./hr.$, though the general level of respiration is falling. Taking recent experiments consecutively, I have calculated in 28 cases the average variation from the mean over the period 1–2 hr. for the separate half-hours, and have obtained the value $\pm 7\cdot8 \mu l.$; this is only a small percentage of the total values involved. For the period $0\cdot5$–$1\cdot0$ hr. the average difference from the same mean is greater, namely $\pm 15\cdot2 \mu l.$ Hence there is experimental justification for the idea that the system once formed remains stable, and proof that the values for the period 1–2 hr. are more consistent (σ for individual figures varying from 300–600 $\mu l.$ for 1–2 hr. is 22 and for $0\cdot5$–$1\cdot0$ hr. 63).

Further support for the idea that the aneurin system once formed is irreversible is to be found in the failure to obtain substantial catatorulin effects with normal brain mentioned above, and the absence of a catatorulin action in solutions in which normal brain has been previously shaken.

Accepting the hypothesis that the rate of pyruvate oxidation is proportional to the quantity ap, theoretically this rate should be exactly proportional to the amount of aneurin added, provided that the active surface adsorbs the aneurin practically quantitatively. Hence, up to a certain limit which is set by the amount of the residual aneurin system still in the avitaminous tissue, increases in rate due to equal increments of aneurin should be equal. There should be a straight line relation between the amount of vitamin and the catatorulin O_2 up to a maximum, which should then at once show no further increase. Fig. 2 (Theory) indicates the kind of curves which would be obtained if this view were correct. The typical experimental curves also shown in Fig. 2 are in general agreement with the hypothesis; they indicate that after the addition of $1\cdot0\gamma$ there is in all cases an abrupt discontinuity; in many cases there is practically no increase after $0\cdot5\gamma$. This is consistent with slight variations in the residual vitamin left in different brains and it means that in practice the part of the curve from $0\cdot5$–$1\cdot0\gamma$ is dangerous to use for any but the most approximate estimations. Inspection of the experimental curves obtained during the last few years showed
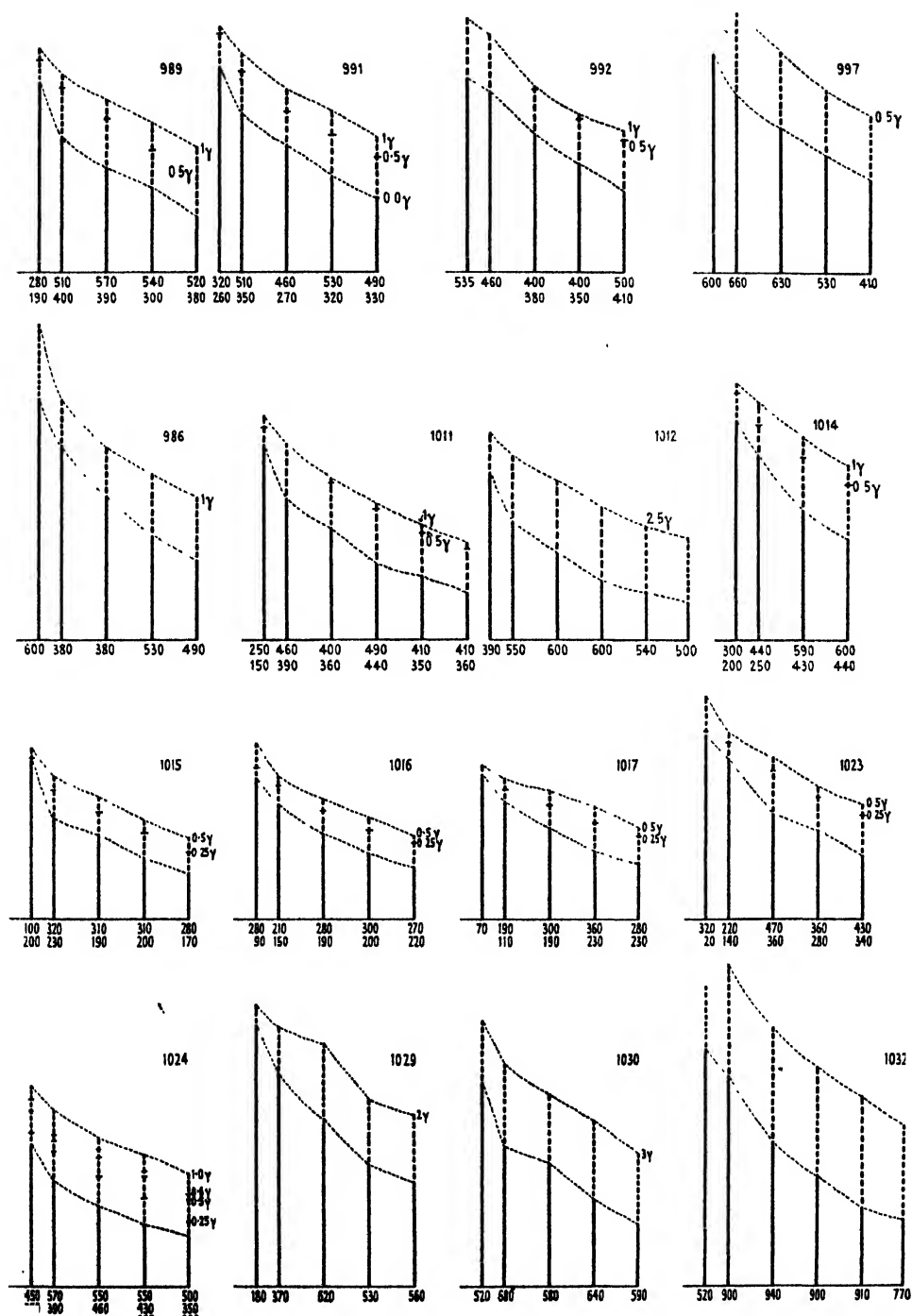
Fig. 1. Catatorulin tests indicating the variation of the respiration rate with time and different amounts of vitamin, indicated as γ on the diagram. Ordinate = rate of respiration, abscissa = time. Each vertical line represents the rate $\mu l./g./hr.$ of respiration for successive periods, 15, 15, 30, 30, 30 min. The figures below the vertical lines represent the extra O_2 uptake due to vitamin.

several which departed more widely from the hypothesis above than could be attributed to experimental error. This departure is always in the sense that the 0.25γ point is higher than it should be for the straight line relation. Owing to the difficulty of standardizing conditions exactly in this system, it is not worth pursuing this point far; the best values for the catatorulin test will be obtained
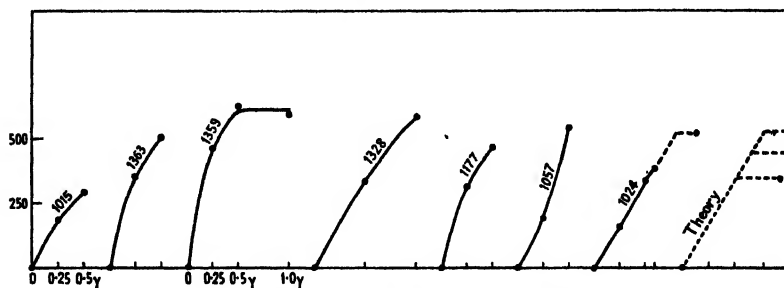
Fig. 2. Curves indicate the change in extra O_2 uptake produced by vitamin in varying concentration. Ordinate = O_2 uptake μl./g./hr.; abscissa = concentration of vitamin; the actual amount is indicated in two experiments; the remainder are drawn to the same scale. Last diagram marked "Theory" is drawn from theoretical considerations.

between 0.25 and 0.5γ, in the region of 0.3γ. I have found, however, that large differences are caused in catatorulin values by not allowing sufficient time for diffusion equilibrium to occur before introducing the bottles into the warm bath; an extreme example of this effect is given in Table I.

Table I. *Effect of allowing time for diffusion on the catatorulin test*

After division of the tissue at the mashing stage, the vitamin was immediately introduced into the bottles. Samples marked "No standing" were quickly filled with O_2 and placed in bath; those "Stood 15 min." were kept at room temperature for this time with shaking.

Extra O_2 uptake due to vitamin for period 1–2 hr., μl./g./hr.

Vitamin added	0.25γ	0.5γ
No standing	556	620
Stood 15 min.	343	523

Here the effect of standing has been to reduce the total O_2 uptake but to improve the divergence between the two concentrations of vitamin. The theoretical straight line is found when the latter values are plotted.

The improvements in method given below are based upon the above principles.

It is not easy to understand why the Michaelis type of equation, proposed by Passmore et al. [1933], held good for lactate. Peters et al. [1935][1] found that the pyruvate formed from lactate in presence of vitamin immediately underwent further oxidation. The most feasible explanation seems to be that the deciding factor was the instability of the pyruvate oxidase system in the absence of pyruvate.

Some experimental points

The procedure should follow that of Kinnersley et al. [1935] (Appendix, p. 712), with the following modifications. The birds should be thoroughly depleted of vitamin by dosing them at least once after symptoms have appeared and using them upon the reappearance of symptoms. Only the cerebrum and

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[1] There was a misprint on page 66 of this paper. In Table II after the arrow in the heading, it should read "poison" addition instead of "substrate" addition.

optic lobes are used. The bottles should contain Ringer-phosphate solution at pH 7·3 and 6 mg. sodium pyruvate before the introduction of the samples. Tests showed that it made no difference whether the pyruvate was added before or after the mashing; it is more convenient to add it before. Triplicate estimations are made if possible, and there should be an attempt to get the points 0, 0·25 and 0·5γ with pure vitamin. The zero point can be obtained in duplicate if only a few samples are available. After division with the glass crusher, which should be carefully done, the vitamin should be added to form the last addition to the bottle soon after the crushing. In this way the concentration is not even temporarily larger than is intended. After the addition of vitamin the bottles should be allowed to stand at room temperature for about 10 min. before filling them with O$_2$ with sufficient shaking to ensure that they do not become anaerobic. An extra shake after filling them with O$_2$ is desirable. I have found it convenient to place the bottles in the warm bath at 38° at intervals of two each $\frac{1}{2}$ min.; this allows reasonable time for reading. The calculations are made by plotting the average for the extra O$_2$ uptake on squared paper for the period 1–2 hr. and reading the value for the unknown by inspection. As a convention I have usually joined the points 0, 0·25 and 0·5γ by straight lines when the 0·25γ point has not lain upon the direct line between 0 and 0·5γ. In some cases it would be unquestionably correct to continue a straight line through the points 0 and 0·25γ and assume that this becomes discontinuous at some point between 0·25 and 0·5γ; but the possibility that there is more initial vitamin than the equivalent of 0·5γ in the tissue has to be balanced in such cases against the possible error of determining the points 0 and 0·25γ in question. The standard deviation of a single observation is of the order of 3·5% of the mean. It is not always easy to get more than ten satisfactory samples from a given brain; the mixing of two avitaminous brains has not proved an advantage owing to the increasing standard deviation found for individual samples. Hence the improvement with triplicate estimations must be balanced against the fewer determinations possible. The theoretical error for an estimation using triplicate observations is of the order of $\pm 5\%$. It is difficult to give an exact idea of the error of the method in practice, because it is so much affected by the condition of the brain used; the results from some brains have to be discarded.

Exps. 1 and 2 below indicate the kind of results which may be expected and which are often obtained; but it must be emphasized that, as the method is subject to unaccountable variations, it is unwise to rely upon less than three estimations.

Exp. 1. Two tests were made in which 0·3γ of a vitamin solution was compared against 0·0, 0·25 and 0·5γ. The values found by estimation were 0·32 and 0·33γ.

Exp. 2. Two synthetic specimens of vitamin B$_1$, now known to be equally active, were tested at a level of 0·5γ. The mean O$_2$ uptakes during the period 1–2 hr. in duplicate estimations were in μl./g./hr.: specimen 1, with 0·0γ, 656 and with 0·5γ, 1027; specimen 2, with 0·5γ, 1063 and with 0·0γ, 654. The two separate values with 0·0γ agree well. The excess O$_2$ values of 371 and 409 give a value for specimen 2 of approx. 0·55γ. The extrapolation seems to be justifiable in this case; but the experiment was an early one, done before it was realized that 0·5γ was practically the maximum amount which could be estimated by this method.

The sodium pyruvate used was prepared as advised by Dr Stedman. I have found it best to dilute redistilled pyruvic acid with about an equal volume of water, add bromophenol blue and neutralize with concentrated pure NaOH to pH 4·5 approximately.

After cooling in ice-salt mixture, the salt is thrown out with acetone. Recrystallization was best done by dissolving in a minimum amount of water, treating with acetone until the appearance of strong turbidity, warming until the whole had just dissolved, and cooling in an ice-salt mixture. Purity was checked by estimation of the bisulphite-binding power.

SUMMARY

The catatorulin test using avitaminous pigeon brain has been studied further; it may still occasionally be useful owing to its specificity for small amounts of vitamin B_1 (about 0.2γ).

Several improvements have been introduced; pyruvate is substituted for lactate. An explanation has now been found for the discontinuous type of curve found with pyruvate and the upper limits of sensitivity have been defined.

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CCLXIV. THE SUGAR AND TOTAL KETONE CONTENT OF THE BLOOD OF EWES AND OF THEIR NEW-BORN LAMBS

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IN many animals it has been found that the maternal blood at term contains a higher percentage of sugar than the foetal blood. Needham [1931] cites data illustrating this point and gives figures obtained in human subjects by 18 investigators, from which the following averages have been calculated: maternal blood 118 mg., foetal blood 94 mg. sugar per 100 ml. Aron [1924] records even wider differences for the dog (maternal 110 mg., foetal 65 mg. per 100 ml.) and for the guinea pig (maternal 107 mg., foetal 60 mg. per 100 ml.). On the other hand he gives data for the cow (maternal 100 mg., foetal 110 mg.) and for the pig (maternal 100 mg., foetal 139 mg.) which suggest that the reverse is true for these species. Passmore & Schlossman [1938] record a similar relation for sheep and goats.

In the course of a study on the occurrence of ketonaemia in pregnant ewes the opportunity was taken to determine the sugar and total ketones present in the blood of ewes (both normal and ketonaemic) at the time of parturition and in that of their new born lambs. A number of ewes were available which, for some weeks during the latter half of pregnancy, had shown progressively increasing ketonaemia accompanied by marked hypoglycaemia. The lambs from these ewes were small and weak and it was of interest to see whether this weakness could be correlated with a low blood sugar level and/or the passage of ketones from the maternal to the foetal blood stream. As controls a number of healthy ewes with their vigorous new-born lambs were also examined.

METHODS

Blood sugar: Somogyi's modification of the Shaffer-Hartmann method [Peters & Van Slyke, 1931].

Total ketone bodies in the blood: Gravimetric method using Denigès' reagent [Peters & Van Slyke, 1931].

RESULTS

All the ewes were bled a day or more prior to parturition and as soon after lambing as possible, the new-born lambs being bled at the latter time. The analytical data are recorded in Table I with the times before and after lambing at which the samples were taken. They serve to show the changes which occur in the blood of the ewe at about the time of labour as well as the differences between the blood of the new-born lamb and its dam with respect to sugar and ketone bodies.

Blood sugar. In the normal healthy ewe at parturition there is a marked rise in the blood sugar level, but the figures cited suggest that *post partum* there is a fairly rapid return to the normal level. Thus, although the ewes bled up to within

Table I. *The sugar and total ketone bodies in the blood of ewes at parturition and in that of their new-born lambs: mg. per 100 ml.*

Ewe no.	Days before lambing	Ewe				Lamb		Interval between lambing and bleeding
		Before lambing		After lambing				
		Sugar	Ketones	Sugar	Ketones	Sugar	Ketones	
				Healthy ewes				
40	1	52	0	145	0	163	0	10 min.
65	6	56	0	112	0	156	0	30 ,,
68	2	60	0	83	0	109	0	2½ hr.
44	1	56	0	72	. 0	94	0	3 ,,
79	3	50	0	50	0	97	0	14 ,,
				Ewes showing ketonaemia				
81	3	18	48	62	25	62	0	5 min.
48	1	40	36	55	16	65	0	5 ,,
41	8	32	41	—	44	—	0	5 ,,
4	1	31	83	67	60	124	0	15 ,,
89	4	38	12	46	12	64	0	15 ,,
37	6 hr.	39	28	37	24	92	0	30 ,,
98	6	28	48	64	32	93	0	60 ,,
11	8	28	53	53	33	68	0	60 ,,

3 hr. after lambing showed this rise, in the case of ewe no. 79 which was not bled until 14 hr. after lambing, the blood sugar was at the same level as it had been 3 days before lambing. A similar rise in blood sugar level at term has been reported in the case of human subjects by Ketteringham & Austin [1938] who found that at the first stage of labour (1–8 hr. before delivery) 28 mothers had an average blood sugar of 93 mg. per 100 ml. and at delivery the average value was 122 mg. per 100 ml. In the ewes suffering from ketonaemia and having a low blood sugar level before lambing the rise was also shown but the level reached was by no means as high as in the healthy ewes.

In the case of the lambs it is shown that at birth, or shortly after, their blood contained a higher percentage of sugar than did that of the mother. This difference is pronounced in the case of the healthy ewes and their progeny, but is less marked for the lambs from some of the ketonaemic ewes. It is probable that, following birth, the blood sugar levels of the lamb may change with rapidity over a fairly wide range. Thus Passmore & Schlossman [1938] found a rise in the blood sugar from 128 to 142 mg. per 100 ml. in the 15 min. following the delivery of an experimental lamb which had been treated with insulin. They suggest a *post partum* mobilization of liver glycogen as a result of the exposure of the new-born lamb to a cold atmosphere. If this elevation is general the rather low blood sugar levels obtained 5 min. after birth could be explained and the rather higher values obtained soon after might be expected. Ketteringham & Austin [1938] found that 3–6 hr. after birth the blood sugar of human babies dropped to the normal infant range. The results shown for the lambs from normal ewes suggested that an analogous change could be demonstrated in the lamb. The smaller differences between the blood sugar levels of the ketonaemic ewes and their lambs and the smaller changes in the blood sugar level of these lambs at the longer intervals from the time of birth are in all probability attributable to the hypoglycaemic condition of the ewe during the latter stages of pregnancy.

Blood ketones. An interesting finding of this investigation is the complete absence of ketone bodies from the blood of the new-born lambs from ewes which had been showing signs of severe ketonaemia for periods up to 6 weeks prior to and immediately after lambing. One might expect that molecules of the size

and nature of β-hydroxybutyric acid (the chief ketone present in the blood of the ketonaemic ewe) would pass readily through the placental membrane. Many investigators have shown that in most mammals gases and crystalloid substances with small molecules pass readily through the placenta. Thus Anselmino [1929] found that 50 min. after injecting dihydroxyacetone into the blood stream of the pregnant rabbit, the foetal and maternal bloods contained the same concentration of this added material. Needham [1931] discusses the variation in permeability shown by different types of chorion and gives as an illustration the fact that sodium salicylate will pass readily through the placenta of the cat or the guinea-pig but not through that of the cow or ewe. It seems that the placenta of the ewe likewise prevents the passage of ketone bodies to the foetus.

It is possible that the foetal lamb possesses some mechanism facilitating the oxidation or removal of ketone bodies from the system, but this does not seem probable. It is generally accepted that young animals develop ketosis more readily than adults and suffer more severely from its effects. This does not suggest the existence of any special protective mechanism in the young.

Summary

1. The sugar and total ketones in the blood of normal and ketonaemic ewes prior to and shortly after lambing are recorded along with the corresponding figures for the blood of the lamb, drawn shortly after birth.

2. At parturition there is a marked rise in the blood sugar levels of both normal and hypoglycaemic ewes.

3. The blood of the new-born lamb contains a higher percentage of sugar than that of its dam bled at the same time.

4. Hypoglycaemia in the pregnant ewe is reflected in a much lower blood sugar level at parturition and a relatively low blood sugar level in the new-born lamb.

5. The blood of new-born lambs from ewes showing marked ketonaemia was quite free from ketone bodies.

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CCLXV. THE SPECIFICITY OF COUPLED ESTERIFICATION OF PHOSPHATE IN MUSCLE

BY DOROTHY MOYLE NEEDHAM AND GWEI DJEN LU

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IT has been shown that, while a number of oxidoreductions involving oxidation of triosephosphate to phosphoglyceric acid can be coupled with synthesis of adenylpyrophosphate from adenylic acid and free phosphate, similar oxidoreductions involving oxidation of glyceraldehyde to glyceric acid are not so coupled [Needham & Pillai, 1937]. Further experiments, in which a variety of conditions have been tried, have confirmed this lack of coupling. From these observations the inference may be drawn that the essential energy-providing reaction in the coupled mechanism is the reduction of coenzyme I by glyceraldehydephosphate; for the oxidation of reduced coenzyme by pyruvate goes on (when glyceraldehyde is the reductant in the oxidoreduction) without coupled esterification. As this inference must be important in the elucidation of the mechanism of the coupling, it was decided to test it further.

This has been done by separating the oxidoreduction into its two constituent reactions:

(1) glyceraldehydephosphate + coenzyme
$$\rightarrow \text{phosphoglyceric acid} + \text{reduced coenzyme},$$

(2) pyruvate + reduced coenzyme $\rightarrow$ lactic acid + coenzyme,

and testing each separately for its effect on esterification. The experiments with reaction (2) are quite straightforward, but with reaction (1) a complication arises. In muscle extract it has not so far been possible to separate the glycerophosphate dehydrogenase from the triosephosphate dehydrogenase; this means that, in aqueous muscle extracts or in extracts of acetone powder, triosephosphate acts as oxidant as well as reductant, and the coenzyme reduced during phosphoglyceric acid formation is oxidized by more triosephosphate, which is itself reduced to glycerophosphate. We have therefore studied the triosephosphate oxidation to phosphoglycerate under conditions where the reduced coenzyme was largely, if not entirely, reoxidized by atmospheric O_2 through the cytochrome system. To enable O_2 uptake to go on, it was necessary to add to the extract of muscle acetone powder ordinarily used for study of oxidoreductions, a small amount of an enzyme preparation from heart made according to the method of Keilin & Hartree [1938]. This preparation contains (1) coenzyme factor, the enzyme catalysing the transfer of hydrogen from reduced coenzyme I or II to a carrier such as cytochrome [Dewan & Green, 1938]; (2) cytochromes a and b; (3) cytochrome oxidase, the enzyme catalysing the oxidation of reduced cytochrome by atmospheric O_2 [Keilin & Hartree, 1938]. When this heart

preparation is added to muscle extract, the following series of reactions can take place:

$$\text{Triosephosphate} + \text{coenzyme I} \xrightarrow{\text{dehydrogenase}} \text{phosphoglycerate} + \text{reduced coenzyme.}$$

$$\text{Reduced coenzyme} + \text{cytochrome} \xrightarrow[\text{factor}]{\text{coenzyme}} \text{coenzyme} + \text{reduced cytochrome.}$$

$$\text{Reduced cytochrome} + O_2 \xrightarrow[\text{oxidase}]{\text{cytochrome}} \text{cytochrome.}$$
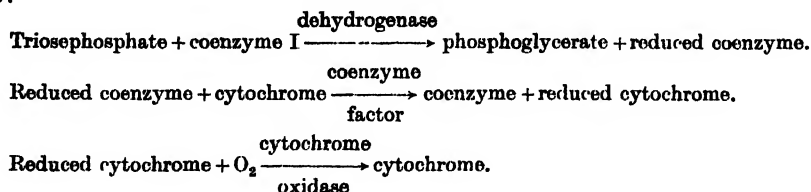
The amount of cytochrome present in our preparations was not optimal, and greater O_2 uptake was always obtained by adding methylene blue. Then

$$\text{Reduced coenzyme} + \text{MeB} \xrightarrow{\text{coenzyme factor}} \text{coenzyme} + \text{reduced MeB.}$$

$$\text{Reduced MeB} + O_2 \longrightarrow \text{MeB.}$$
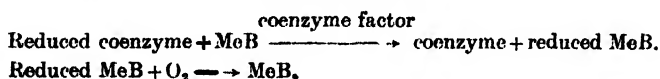
EXPERIMENTAL DETAILS

Enzyme preparations. The aqueous extract of dried acetone muscle powder (used in all the experiments to provide the oxidoreduction and phosphorylation mechanisms) was prepared in the same way as described by Needham & Pillai [1937]. Before use the extracts were dialysed 18 hr.

The heart preparation (added when interaction with atmospheric O_2 was needed) was made from pig's heart in the following way. Two hearts were freed from fat and minced twice through an ordinary mincer; the mince was thoroughly washed by suspending (with mechanical stirring) in about 5 l. tap water; this was repeated ten times, the muscle mass being squeezed in muslin each time before resuspension. It was then ground with fine sand and 250 ml. $M/25$ phosphate buffer (pH 7) for $1\frac{1}{2}$ hr. in a mechanical mortar. The thick paste was mixed with 350 ml. more buffer solution and centrifuged. The cloudy fluid was poured off, and acetate buffer pH 4·6 was added until the pH of the whole had fallen to 4·6. The mixture was again centrifuged; the clear fluid was discarded, and the residue was suspended in 30 ml. phosphate buffer ($M/10$ pH 7·2). Finely powdered Na_2HPO_4 was added until the pH was 7·0. The preparation was stored at 0° in this form; at the end of a week its activity was about 60% of the initial. Before use, portions of the preparation were dialysed 18 hr. in cellophane bags suspended in 3 l. 0·5% KCl.

It was already known that the extract of acetone muscle powder contained no adenylpyrophosphatase and no deaminase. As the heart preparation also was to be used in experiments where adenylpyrophosphate synthesis from adenylic acid was to be looked for, it was necessary to find out in this case too whether enzymes were present which could dephosphorylate synthesized adenylpyrophosphate, or deaminate added adenylic acid.

As regards possible destruction of added adenylic acid, the position appeared satisfactory; for under the conditions of the experiments, not more than 5% of the possible NH_3 was set free in 15 min.

On the other hand, unfortunately, it seemed to be impossible to free the heart preparation from adenylpyrophosphatase. Even after some days at 0° and several hr. dialysis added adenylpyrophosphate was broken down to a considerable extent. In order in these circumstances to demonstrate disappearance of inorganic phosphate, we tried adding to the experimental mixture glycogen as well as adenylic acid, in the hope that phosphate would be transferred to glycogen from synthesized adenylpyrophosphate more rapidly than it was split

off again from the adenylpyrophosphate to give free phosphate. Experiments in which adenylpyrophosphate+glycogen were added to the enzyme mixture showed that, even in the presence of glycogen, rather more than half the pyrophosphate disappearing was found as inorganic phosphate; thus the adenylpyrophosphatase appeared to be more active than the enzyme concerned with phosphate transfer. Creatine (at pH 9·0) instead of glycogen was tried, but with even less success. In absence, therefore, of any better way of demonstrating esterification of free phosphate, glycogen+adenylic acid were used in the O_2 uptake experiments.

The use of glycogen in this way led to the possibility of another source of error: the Parnas esterification of inorganic phosphate with glycogen might take place, leading to disappearance of inorganic phosphate quite independently of the coupled mechanism. Tests such as the following showed, however, that with the concentrations of phosphate, glycogen and enzymes used in the experiments, no Parnas esterification was observable.

Three mixtures were prepared. Each contained glycogen 3·6 mg., adenylic acid 0·6 mg., phosphate (0·066 M, pH 7·2) 0·3 ml., $MgSO_4$ (0·006 M) 0·05 ml. and NaF (0·5 M) 0·1 ml.; water to 2·4 ml. In addition the following were added:

	1	2	3
Heart preparation	0·75 ml.	0·75 ml.	0 ml.
Muscle extract	0·25 ml.	0 ml.	0·25 ml.

An initial sample of 0·5 ml. was removed from each, then all were incubated at 37°. Samples were again removed at the end of 15 min. and 30 min. Only in no. 3 was any change in free phosphate observed, and here the decrease was only 0·09 mg. out of 0·6 mg.

Finally, tests were made to see whether the hexosediphosphatase activity discussed by Pillai [1938, 1] was of importance under our conditions.

Three mixtures were prepared and incubated at 37°. Each contained hexosediphosphate (0·14 M) 0·15 ml., phosphate (0·066 M, pH 7·2) 0·3 ml., $MgSO_4$ (0·006 M) 0·1 ml. and NaF (0·5 M) 0·1 ml.; water to 2·2 ml. In addition the following were added:

	1	2	3
Heart preparation	0·75 ml.	0·75 ml.	0 ml.
Muscle extract	0·25 ml.	0 ml.	0·25 ml.

Samples were removed at 0, 15 and 30 min. The increase in inorganic phosphate found was within the limits of experimental error.

Summing up this preliminary examination of the properties of the enzyme preparations to be used, we may therefore say that no destruction of adenylic acid by deaminase, no significant removal of free phosphate by the Parnas esterification, and no significant increase in free phosphate by hexosediphosphatase activity is to be expected. On the other hand, owing to the persistence of adenylpyrophosphatase in the heart preparation, some hydrolysis of adenylpyrophosphate, even in presence of glycogen, will take place, and one can hope to observe only a fraction of the adenylpyrophosphate synthesis actually occurring.

Chemical preparations and methods. These were for the most part the same as those used in previous work [e.g. Needham & Pillai, 1937]. For estimation of phosphoglyceric acid the method of Rapoport [1937] was followed and for O_2 uptake the method of Warburg, using 15 ml. conical cups with two side-bulbs.

LACK OF ESTERIFICATION WITH GLYCERALDEHYDE OXIDOREDUCTION

In the preliminary experiments of Needham & Pillai [1937] it was found that oxidoreduction between glyceraldehyde and pyruvate went on unaccompanied by esterification of free phosphate when adenylic acid was present as phosphate acceptor. About 1·5 mg. lactic acid per ml. extract were formed; when similar amounts of lactic acid were formed by reduction of pyruvate by triosephosphate, about 0·5 mg. inorganic P disappeared. In the present work these results were confirmed. It seemed possible that the high adenylic acid concentration used (about 3 mg./ml. final concentration) might have some deleterious effect upon the coupled mechanism with glyceraldehyde as a component, although not with triosephosphate. Some experiments were therefore done with glycogen and creatine provided as possible acceptors of phosphate from adenylpyrophosphate if formed, only small amounts of adenylic acid being added.

Typical experiments are given below.

1 ml. extract was made up to a final volume of 3 ml. The final concentrations were: pyruvate, 0·026 M; hexosediphosphate, 0·013 M; inactive glyceraldehyde, 0·052 M (to allow for the l-component, which does not react); phosphate, 0·03 M; NaF, 0·025 M; NaHCO$_3$, 0·01 M. Crude coenzyme was added, 0·3 ml. of a solution containing 150 mg. per 100 ml. All samples were incubated 30 min. at 37°, pH 8·0. The results are expressed in mg. per ml. extract.

Table I

	Lactic acid mg.	Inorganic P mg.	Creatine-phosphate P mg.
Adenylic acid, 0·01 M (3·4 mg. per ml.)			
Hexosediphosphate + pyruvate	+1·2	−0·5	—
Glyceraldehyde + pyruvate	+1·5	0	—
Adenylic acid, 0·002 M (0·7 mg. per ml.)			
Hexosediphosphate + pyruvate + glycogen (6 mg.)	+0·94	−0·27	—
Glyceraldehyde + pyruvate + glycogen (6 mg.)	+0·90	0	—
Hexosediphosphate + pyruvate + creatine (10 mg.)	+1·31	—	+0·17
Glyceraldehyde + pyruvate + creatine (10 mg.)	+1·10	—	0

In the case of glyceraldehyde oxidoreduction with pyruvate, it was noticed that the lactic acid formation was much greater in a sample to which no adenylic acid or phosphate had been added. Further experiment showed that addition of adenylic acid has a marked inhibitory effect on the oxidoreduction, while addition of phosphate has a stimulating effect.

Table II

	Lactic acid formed (mg.)	
	Exp. 45 A	Exp. 45 B
No adenylic acid and no phosphate added	0·43	0·37
Both added	0·84	1·05
Phosphate added, no adenylic acid	1·44	1·24

For hexosediphosphate oxidoreduction with pyruvate the following results were found:

Table III

	Lactic acid formed (mg.)		
	Exp. 46	Exp. 48	Exp. 59
No adenylic acid and no phosphate added	0·84	0·64	—
Both added	1·28	2·4	1·19
Phosphate added, no adenylic acid	1·09	1·54	0·39

Here again the stimulating effect of phosphate addition is seen, but in this case adenylic acid shares in the stimulation instead of opposing it.

In the previous work of Needham & Pillai [1937] a stimulatory effect of higher adenylic acid concentrations (0·02 and 0·03 M) upon the oxidoreduction with triosephosphate was noticed; and Pillai [1938, 2] has found that with very thoroughly dialysed extracts (90 hr.), though oxidoreduction can go on without addition of phosphate or adenylic acid, activation follows on addition of these substances. Meyerhof et al. [1937; 1938] have observed the very marked stimulating effect of adenosinediphosphate upon reduction of coenzyme I by triosephosphate in presence of B protein from yeast, and this has been confirmed by Adler & Günther [1938]. Meyerhof and·his collaborators consider that only when the esterification process is going on can the oxidoreduction between triosephosphate and pyruvate proceed. In view of all these facts the inhibitory effect of adenylic acid upon the uncoupled glyceraldehyde oxidoreduction is interesting.

ESTERIFICATION ACCOMPANYING TRIOSEPHOSPHATE OXIDATION

In these experiments 1 ml. heart preparation and 0·3 ml. acetone powder extract were used in each Warburg cup, with 0·1 ml. 5% methylene blue. The amount of heart preparation was the limiting factor in the O_2 uptake, which was not increased by using more muscle extract or carrier. The total volume was 3–4 ml. The concentration of phosphate was about 0·01 M and its pH 7·2. Crude coenzyme I was added, 0·4 ml. of a solution containing 150 mg. per 100 ml. NaF 0·025 M was always present to prevent breakdown of the phosphoglyceric acid formed. As explained in an earlier section, adenylic acid was used in low concentration as transporter in these experiments, and glycogen was present to accept the phosphate. About 1 mg. adenylic acid and 6 mg. glycogen were present in each cup. To provide the triosephosphate for oxidation, hexosediphosphate was added in amount to give a final concentration of 0·01 M. The neutralized solutions of hexosediphosphate and of adenylic acid were placed in the two side-bulbs, and were tipped in only after temperature equilibration.

Controls showed that (a) without added hexosediphosphate no O_2 uptake occurred; (b) addition of methylene blue increased the O_2 uptake by about 50%; (c) it was very low without acetone powder extract; (d) with acetone powder extract but no heart preparation it was about 20% of the amount when both were present. Iodoacetate (0·003 M) completely inhibited the O_2 uptake with hexosediphosphate.

All samples in Table IV were kept 30 min. at 37°; the results are given in μl. and mg. per cup.

In most of these experiments, no dismutation of the hexosediphosphate has taken place. This is shown by the fact that the increase in difficultly hydrolysable P and the increase in phosphoglyceric acid P are about equivalent to each other and to the O_2 uptake. In a minority of the experiments, however, dismutation as well as oxidation of the triosephosphate took place. This is shown by the fact that the increase in difficultly hydrolysable P is much more than the equivalent of the O_2 uptake. In Exp. 30, for example, where the phosphoglyceric acid P was measured as well, this was found to be greater than the P equivalent of the O_2 uptake by 0·5 mg. This amount therefore must have been formed by dismutation, and the presence of 0·5 mg. glycerophosphate P was to be expected. 2·24 mg. difficultly hydrolysable P were indeed found.

Table IV

Exp.	O_2 uptake $\mu l.$	P equiv. of O_2 uptake mg.	Increase in diff. hydr. P mg.	Phosphoglyceric acid P formed mg.	Change in inorganic P mg.
24	210	0·6	0·52	—	−0·7
25	153	0·43	0·55	—	−0·66
27	468	1·3	1·7	—	−0·71
27	384	1·1	1·41	—	−0·89
31	231	0·64	0·52	0·748	−0·20
31	198	0·55	0·70	0·746	−0·21
31	249	0·68	0·72	0·702	−0·29
32	172	0·48	0·43	0·62	−0·19
32	181	0·50	0·64	0·71	−0·22
35	252	0·73	0·82	1·00	−0·46
37	335	0·93	0·87	1·1	−0·25
37	310	0·86	0·97	1·1	−0·22
37	300	0·84	0·99	1·1	−0·30
64	267	0·74	0·63	—	−0·4
64	429	1·2	0·99	—	−0·75
26	380	1·13	2·205	—	−1·02
26	263	0·77	2·17	—	−0·98
30	389	1·07	2·24	1·54	−0·71

These results on muscle may be compared with those obtained by Lennerstrand & Runnström [1935] and Lennerstrand [1936] on apozymase from dried yeast. Adenylpyrophosphatase seems to be absent from such preparations, for there was good agreement between inorganic phosphate disappearing and phosphoglyceric acid formed. Lennerstrand found that, using phosphate concentrations of about 0·03 M, the O_2 uptake was much less than with higher P concentrations. At a P concentration of nearly 0·3 M it had increased to tenfold. This effect was caused by long persistence of the power of O_2 uptake, the initial rate being about the same at all P concentrations. No correspondingly large effects were observed with muscle. Raising the P concentration from 0·01 to 0·03 M caused a 50% rise in O_2 uptake; but further increase to 0·1 M caused no further rise.

Meyerhof et al. [1938], using "B protein" from yeast (which contains no glycerophosphate dehydrogenase), have recently shown that when triosephosphate reacts with coenzyme I to form phosphoglyceric acid and reduced coenzyme there is equivalent phosphate esterification.

Some experiments were done in which lactate and succinate were added as substrate for oxidation. The experimental conditions were as usual, except that, with lactate, HCN was added to remove the pyruvate formed [Green & Brosteaux, 1936]. No esterification accompanied O_2 uptake in these cases.

Table V

Substrate	O_2 uptake $\mu l.$	Change in inorg. P mg.
Lactate	190	−0·06
Succinate	240	−0·10

LACK OF ESTERIFICATION WITH OXIDATION OF REDUCED COENZYME

In these experiments excess of pyruvate together with a known amount of reduced coenzyme I were added to the extract of muscle acetone powder, in presence of phosphate and adenylic acid. The amounts of reduced coenzyme I, of lactic acid and of inorganic P were estimated at the beginning and end of the incubation time.

The estimation of reduced coenzyme was made by measurement of the height of the absorption curve at 345 mμ, using a Spekker photometer.

The absorption coefficient $\beta = \frac{1}{d} \times \frac{1}{c} \times \ln \frac{I_0}{I}$,

where d = length of tube in cm.

c = concentration of absorbing solute in g. mol. per ml.

$\frac{I_0}{I} = \frac{\text{intensity of light entering tube}}{\text{intensity of light leaving tube}}$.

Warburg et al. [1935; 1938] have shown for the dihydro bands at 345 mμ, characteristic of the nucleotides coenzymes I and II, that

$$\ln \frac{I_0}{I} = 1\cdot 3 \times 10^7,$$

when $d = 1$ and $c = 1$ g. mol. per ml. When $d = 2$ (as in our experiments) and $c = 0\cdot 1$ mg. per ml.

$$\ln \frac{I_0}{I} = \frac{1\cdot 3 \times 10^7 \times 2}{663 \times 10,000}.$$

As the drum readings on the Spekker instrument are engraved to give $\log \frac{I_0}{I}$, not $\ln \frac{I_0}{I}$, the reading to be expected for $0\cdot 1$ mg. reduced coenzyme I per ml. in the 2 cm. tube is

$$\log \frac{I_0}{I} = \frac{1\cdot 3 \times 10^7 \times 2}{663 \times 10,000} \times \frac{1}{2\cdot 3} = 1\cdot 7.$$

Solutions of coenzyme I of unknown concentration were diluted to give a reading of about this value, and the original concentration could then be calculated.

The coenzyme I preparation used was made in the usual way [see Needham & Pillai, 1937]. Tests showed that it contained about 25% of the coenzyme. The reduced coenzyme was prepared from it by treatment with $Na_2S_2O_4$, excess $Na_2S_2O_4$ was oxidized by vigorous aeration and the Na_2SO_3 was removed according to the method of Euler & Hellström [1938]. One experiment is described in detail below.

300 mg. crude coenzyme and 40 mg. $Na_2S_2O_4$ were dissolved in 6·6 ml. 1·3% $NaHCO_3$ which had previously been gassed with a stream of $N_2 + 5\%$ CO_2. The mixture stood at room temp. 1 hr. with the gas stream running. The N_2-CO_2 stream was then exchanged for an air current, which ran for 2 hr. At the end of this time, the mixture was warmed to 80°, a slight excess of $BaCl_2$ was added and it was filtered through a heated funnel. The excess Ba was removed with Na_2SO_4. The final volume was 8·4 ml. Two mixtures were made up in Warburg cups, which were then filled with N_2-5% CO_2 mixture and shaken at 18°, until the gas absorption (due to the decrease in acidity when reduced coenzyme is oxidized) was finished.

Each contained reduced coenzyme solution 2 ml., adenylic acid (0·1 M) 0·2 ml., phosphate (0·05 M, pH 7·2) 0·5 ml., $NaHCO_3$ (3·3%) 0·4 ml. and muscle extract 0·3 ml.; to cup no. 1 pyruvate (0·25 M) 0·2 ml. was also added, while 0·2 ml. water was added to cup no. 2.

At the end of the time the cups were emptied into test tubes and washed out with three successive lots of 2 ml. water. The contents of the tubes were coagulated by placing in a boiling water bath and were then mixed with a small quantity of kieselguhr (ca. 20 mg.) and filtered through paper. The volume of each was made up to 20 ml. 2 ml. were used for phosphate determination, 15 ml. for lactic acid and 1 ml. was diluted to 5 ml. for measurement of the absorption spectra (Table VI).

· Table VI

	Reduced coenzyme mg.	Lactic acid mg.	Phosphate mg.
1	0	1·83	0·87
2	9·4	0·69	0·87

Thus 1·14 mg. lactic acid have been formed from pyruvate, while 9·4 mg. reduced coenzyme (equivalent to 1·25 mg. lactic acid) have been oxidized. The inorganic phosphate remained absolutely unchanged.

In order to make sure that no substance capable of preventing phosphorylation was present in the reduced coenzyme solution used, the following control experiment was carried out. The "control solution" was made by carrying out all the processes involved in reduction of coenzyme, and removal of the $Na_2S_2O_4$ and Na_2SO_3, but without adding coenzyme. Three mixtures were made up. Each contained hexosediphosphate (0·14 M) 0·2 ml., pyruvate (0·25 M) 0·2 ml., adenylic acid (0·1 M) 0·2 ml., phosphate (0·066 M) 0·5 ml., $NaHCO_3$ (1·3 %) 1·0 ml., NaF (M) 0·1 ml., coenzyme solution (0·15 %) 0·2 ml. and muscle extract 0·3 ml. No. 1 contained also 2·0 ml. water, nos. 2 and 3 contained each 2·0 ml. control solution. Nos. 1 and 2 were incubated 10 min. at 37°; then coagulated by placing in boiling water. In the case of No. 3, the tube was placed in boiling water before addition of the enzyme solution, so that the latter was coagulated at once. All were mixed with kieselguhr and filtered as usual, and the inorganic P was estimated (Table VII).

Table VII

No.	Inorganic P mg.	P esterified mg.
1	0·55	0·22
2	0·52	0·25
3	0·77	—

The control solution has thus had no inhibitory effect upon the phosphorylation brought about by oxidoreduction between triosephosphate and pyruvate.

Meyerhof et al. [1938] reached the same conclusion that, in yeast, oxidation of reduced coenzyme by acetaldehyde is not connected with phosphorylation. In their experiments the phosphate disappearance was equivalent to the phosphoglyceric acid formed, as already mentioned, and this was the case whether or not acetaldehyde was introduced to reoxidize the reduced coenzyme.

At the beginning of this work the attempt was made to use the reduced coenzyme solution for experiments like that in Table VI without removing the Na_2SO_3 formed on aeration. But experiments on the lines of Table VII showed that these "control solutions" containing Na_2SO_3 had a very marked inhibitory effect upon phosphorylation brought about by oxidoreduction between triosephosphate and pyruvate; the actual oxidoreduction, as measured by lactic acid formation, was unaffected.

Table VIII shows a comparison of the effects of the "control solution" (10 mg. $Na_2S_2O_4$ in 8 ml. 0·1% $NaHCO_3$; well aerated), Na_2SO_3 solution (0·014 M) and arsenate solution (0·014 M). All the samples contained coenzyme, pyruvate, hexosediphosphate, adenylic acid, phosphate, muscle extract and NaF as in the experiment of Table VII.

Table VIII

	Lactic acid formed mg.	P esterified mg.
1. +3 ml. 1% $NaHCO_3$	1·2	0·35
2. +3 ml. control solution	0·94	0·19
3. +3 ml. 0·02 M arsenate in 1% $NaHCO_3$	1·50	0
4. +3 ml. 0·02 M Na_2SO_3 in 1% $NaHCO_3$	1·09	0·19

This effect of sulphite is being further investigated.

SUMMARY

1. Further evidence has been obtained showing that in muscle extract oxidoreductions involving glyceraldehyde are not accompanied by coupled esterification of phosphate.

2. The oxidoreduction between triosephosphate and pyruvate has been separated into its two constituent reactions. The oxidation of triosephosphate to phosphoglyceric acid by coenzyme is accompanied by esterification; the oxidation of reduced coenzyme thus formed by pyruvate is not accompanied by esterification.

3. Adenylic acid has an activating effect on oxidoreduction involving triose-phosphate, but an inhibitory effect on oxidoreduction involving glyceraldehyde.

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CCLXVI. THE CATALYTIC EFFECT OF BUFFERS ON THE REACTION $CO_2 + H_2O \rightleftharpoons H_2CO_3$

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FAURHOLT [1925, 1] has shown that in aqueous solution CO_2 takes part in two independent reactions

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^-, \qquad \ldots\ldots(1)$$
$$CO_2 + OH^- \rightleftharpoons HCO_3^-. \qquad \ldots\ldots(2)$$
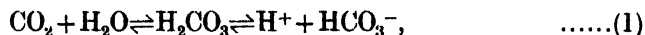
The velocity constant of the reaction $CO_2 + OH^- \to HCO_3^-$ is more than a million times greater than the velocity constant of the reaction $CO_2 + H_2O \to H_2CO_3$, the loss of the proton from the H_2O molecule apparently causing, as Faurholt has emphasized, an enormous increase in the affinity for CO_2. In consequence of this, it is only below pH 8 that the rate of (2) becomes negligible in comparison with the rate of (1): in the range pH 9–10 the two rates are of the same order, whilst above pH 10 the rate of (2) becomes predominant.

Similar studies on the rate of combination of CO_2 with very weakly acidic organic hydroxides, such as methyl alcohol, ethyl alcohol and glucose, have shown [Faurholt, 1927] that CO_2 reacts slowly and in an analogous way with the organic hydroxide molecule:

$$CO_2 + HOX \rightleftharpoons HCO_3 X. \qquad \ldots\ldots(3)$$

The inhibitory effect of the proton is again shown by the fact that the reaction with the organic anion—

$$CO_2 + OX^- \rightleftharpoons CO_3 X^- \qquad \ldots\ldots(4)$$

—is also very much more rapid and complete than the reaction with the molecule.

In the case of stronger oxy-acids the affinity of the anion for H^+ is correspondingly lower, and Faurholt tacitly assumed that the affinity of the anion for CO_2 would fall *pari passu* and hence that acids with pK of the order of 7 (e.g. cacodylic acid) could be safely used as buffers in the measurements of the velocity constants of the reactions $CO_2 + H_2O \rightleftharpoons H_2CO_3$ and $CO_2 + OH^- \rightleftharpoons HCO_3^-$, without fear of the buffer taking any part other than the "instantaneous" supply or removal of the H^+ ions involved in the ionization of carbonic acid, i.e. $H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$. The possibility of any direct reaction of the buffer with CO_2, H_2CO_3 or HCO_3^- (except in the case of the borate ion, which Faurholt suggested might form some special complex with CO_2) was thus implicitly excluded, not only by Faurholt but by all others who have worked in this field.

Preliminary experiments on the activity of carbonic anhydrase in phosphate solutions of varying concentration and pH have, however, prompted us to examine more critically the role of the buffer in reactions (1) and (2). To this end we have measured manometrically both the rate of CO_2 uptake by buffer solutions shaken violently with CO_2, and the rate of CO_2 output from bicarbonate buffer mixtures, in the presence of a much wider range of buffer concentration

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and species than heretofore. Our results, especially those in which the buffer concentration is varied 10-fold or more, lead us to conclude that almost all common oxy-acid-salt mixtures which buffer above pH 6, not only promote the CO_2 reactions by supplying or removing H^+ ions, but also through their more negative constituents catalyse directly both phases of the reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$. A preliminary account of these results has already appeared [Booth & Roughton, 1938]. Faurholt's discovery of the activity of the borate ion is thus only a special, though pronounced, example of a general principle.

It was not until nearly the end of the research that we investigated the effect of nitrogen bases as buffers. We were deterred from doing so by the fact that straight-chain nitrogenous bases, such as NH_3 or $CH_2NH_2COO^-$, combine rapidly and reversibly with CO_2 to form carbamino compounds, according to the following scheme [Faurholt, 1925, 2]:

$$RNH_2 + CO_2 \rightleftharpoons RNH.COOH, \qquad \bullet \qquad \dots\dots(5)$$

$$RNH.COOH \rightleftharpoons H^+ + RNH.COO^-, \qquad \qquad \dots\dots(6)$$

$$H^+ + RNH_2 \rightleftharpoons RNH_3^+. \qquad \qquad \dots\dots(7)$$
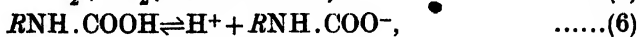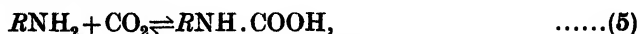
(The inhibitory effect of the proton is shown in this case also by the absence of reaction between CO_2 and RNH_3^+.) The simultaneous occurrence of this carbamino reaction might make it difficult to detect and measure accurately any catalytic effect on the $CO_2 + H_2O \rightarrow H_2CO_3$ reaction.

Cyclic nitrogenous bases have not, so far as we know, been investigated as regards their carbamino-forming power, and it was therefore with special interest that we tested some members of the new range of glyoxaline buffers, recently described by Kirby & Neuberger [1938], to whom we are greatly indebted for samples. Both the cations and the molecules of these substances were found to be incapable of any carbamino reaction with CO_2, but on the other hand the molecules proved to be markedly catalytic towards the reaction $CO_2 + H_2O \rightarrow H_2CO_3$ especially if their pK were greater than 7·0. ($pK = -\log_{10}K$ where $K = [H^+] \times$ [molecule of base]/[cation of base].) This led us to test such other cyclic nitrogenous bases as were both readily available and sufficiently soluble in water. Similar catalytic activity was always found if the pK were greater than 7, whereas tests of a more extended range of straight-chain N bases showed that these were without catalytic effect, their only reaction with CO_2, if any, being the carbamino formation. These rather remarkable results are summarized in Section II, together with some preliminary data on sulphhydryl and other compounds.

A possible mechanism of these catalyses is given in the discussion, together with some further points of interest which arise in regard to the enzyme carbonic anhydrase.

Methods

Rates of CO_2 uptake were determined by an improvement of Meldrum & Roughton's [1933, 2] manometric boat method. The apparatus (Fig. 1) consists of a lagged tank, a stationary manometer containing toluene coloured with sudan III as gauge liquid, a compensating bottle E (about 200 ml.), a CO_2 reservoir D (about 200 ml.) which can be connected through fine thermometer tubing R to a 60 ml. long-necked glass "boat" and a mechanism, not shown, for shaking the boat about AR as axis at an adjustable speed. The shaking must be rapid enough not to limit the rate of CO_2 uptake, but not so violent as to splash solution up into the tube connecting with the manometer. At 315 oscillations per min. the limiting speed is exceeded without the readings becoming erratic.

This speed is kept constant by a synchronous motor and is indicated by a neon lamp stroboscope.

The procedure in an experiment is as follows. The tank is emptied, and dried air is drawn through the connecting tubes to remove condensed moisture. In the meantime the test solution is measured into the "boat", which is stoppered and pushed into a holder in the shaker which holds it firmly by means of two rubber sleeves encircling the boat, all clips being open except C_4. The tank is then filled with water and the stirrer turned on. D is repeatedly evacuated and filled with CO_2 and left with a CO_2 pressure of 30–40 cm. Hg, the clips being closed and water-sealed. The boat, compensating bottle and manometer are evacuated to $\frac{1}{2}$ atm. through C_6 which is closed and placed below the water surface. After 9 min. the boat is shaken for 2 min. to bring the dissolved gases
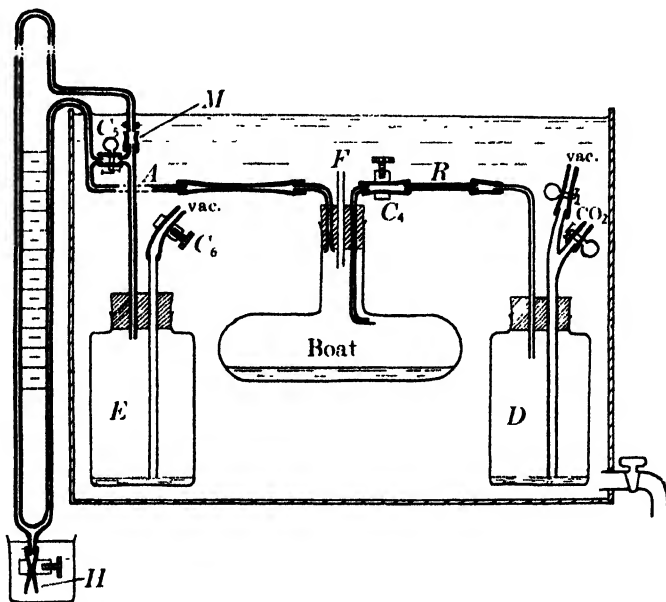
Fig. 1. Simplified diagram of apparatus arranged for CO_2 uptake. Not to scale. The boat is oscillated through 50° at right angles to the plane of the paper. Tube F is only used for anaerobic experiments.

in the test solution into equilibrium with the gas phase, and 1 min. is allowed for the solution to drain to the bottom of the boat. The short circuit C_5 between the two limbs of the manometer is closed and the zero reading taken. CO_2 is slowly let into the boat through the resistance R, by manipulation of C_4, up to the desired pressure. The time taken by this should be about 30 sec. A further period is allowed for the disturbance caused by the admission of the CO_2 to settle down, and then, exactly 2 min. after CO_2 begins to enter, shaking is started and manometer readings taken at 0, 5, 10, 15 sec. etc., up to 5 min. or more. The tank temperature is read. At the end of the experiment the clip C_5 is opened, and air allowed to enter slowly through a capillary inserted at C_6 until all parts of the apparatus are at atmospheric pressure. (If air enters suddenly into the apparatus, when at low pressure, the gauge liquid will shoot over.) Most of the water is meanwhile emptied out of the tank, and the boat then removed, washed and dried.

The preliminary equilibration period is 12 min.; if duplicate boats are used and one is prepared during the equilibration of the other, an observer and an assistant can perform serial experiments at the rate of one every 25 min.

With axial shaking, only a slight vibration is transmitted to the manometer and volume variations from distortion of the thick rubber tubing are negligible. Furthermore all the rubber joints can be water-sealed, the dead space between the boat and the liquid meniscus in the manometer cut down to 1 ml., and the volume of the system exposed to room temperature reduced to about 0·2 ml. These improvements, together with the substitution of toluene for water in the manometer gauge, the modified boat shape and the constant speed motor, are responsible for the increase in accuracy and reliability of the method. When the apparatus is working properly, readings should duplicate to 0·2 mm. At temperatures other than that of the room stirring of the water in the tank must be vigorously maintained during equilibration; otherwise the temperature, and therefore the pressure, in the compensating bottle or boat may vary from one another.

From time to time the whole apparatus must be washed out with water and acetone and dried by flushing with dried air. The manometer is then refilled with de-aerated coloured toluene through the rubber tube H. This tube is then filled with de-aerated water to form a seal and immersed in water. A little water is always left in E to saturate the gas with vapour.

When autoxidation, leaks or other complications which would give spurious results by manometric methods were suspected, readings were taken in absence of CO_2 during a prolonged first shaking period. For autoxidizable substances experiments were done under anaerobic conditions, by applying a slight modification of the method used by Meldrum & Roughton [1933, 2] for studying the rate of uptake of CO_2 by reduced blood to the present technique. If necessary one component of the required solution could be weighed solid directly into a dry boat, the oxygen removed by washing out with nitrogen and the remaining de-aerated solution added from a tonometer through F. In this case the substance makes no contact with oxygen whilst in solution.

Unless otherwise stated all experiments by the CO_2 uptake method were done at 0° with 4·2 ml. solution and an initial CO_2 pressure of 12·8 mm. Hg in a total gas space of 60 ml.

In order to avoid the effect of complications due to the $CO_2 + OH^- \rightarrow HCO_3^-$ reaction, most experiments were done below pH 8·0.

Rates of CO2 output were determined in the same apparatus but with the double compartment boat method of Meldrum & Roughton [1933, 1]. After equilibration of the boat in the tank the solutions were suddenly mixed (by starting the shaker) and the CO_2 evolution followed manometrically. Most of the experiments were done at 0° and at a total gas pressure of $\frac{1}{3}$ atm.

The majority of the experiments were done with the illustrated apparatus which has however now been simplified. The outlet clip, water-seal etc., at H have been eliminated and the gauge fluid is introduced into the manometer tube through M.

Notes on solutions. Carbonic anhydrase was prepared in semi-purified state by Meldrum & Roughton's [1933, 1] chloroform method.

Buffer solutions were made up with a known acid/ion ratio by adding the calculated quantity of HCl or alkali, and checking the pH or titrating to an unbuffered end-point. The pH of solutions was determined in most cases colorimetrically but for special cases it was measured by the hydrogen electrode.

Na and K salts were used indiscriminately, except when high concentrations were required and one salt (usually K) is markedly more soluble than the other.

I. Oxy-acid buffers

The two buffers which have been most used hitherto in work on CO_2 kinetics are phosphate $(H_2PO_4^- + HPO_4^-)$ and cacodylate $(HCac + Cac^-)$, where Cac^- is

$$CH_3 \diagdown$$
$$CH_3—As \doteq O.$$
$$-O \diagup$$
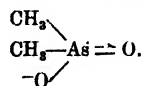
We have accordingly investigated these with special care, and since the results and inferences therefrom seem qualitatively the same as for the general class of oxy-acid buffers which function above pH 6, we shall first describe the results with phosphate and cacodylate in detail.

CO_2 uptake experiments

Curve A, Fig. 2, shows the rate of CO_2 uptake by water, recorded manometrically. The gaseous CO_2 pressure drops to a steady value in about 20 sec.: this rapid uptake is simply due to physical solution of CO_2, the subsequent H_2CO_3 and HCO_3^- formation being negligible.

Curves B, C, D, Fig. 2, show the effect of increasing concentration of total phosphate, the ratio of $[HPO_4^-]$ to $[H_2PO_4^-]$ being kept constant. In each case there is a similar rapid drop of CO_2 pressure in the first 20 sec. (though the drop may be less owing to the solubility of CO_2 in the buffer being lower than in water), and then a prolonged slow phase in which the dissolved CO_2 enters into chemical combination. The slope of this slow phase is seen to be steeper
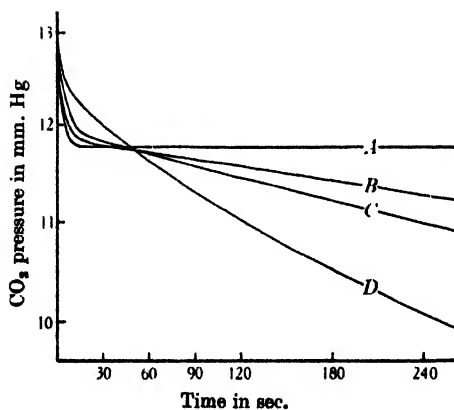
Fig. 2.

Fig. 3.

Fig. 2. Curves showing the observed course of CO_2 uptake by water and by phosphate at 0°. A, water; B, 0·0095 M phosphate; C, 0·14 M; D, 0·76 M. The pH was 7·4 at 0·2 M.
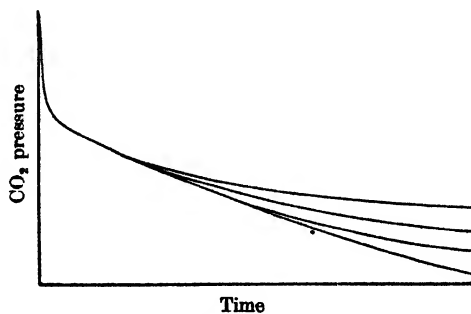
Fig. 3. Curves showing the expected course of CO_2 uptake by various concentrations of a given buffer, if the buffer itself has no direct effect on the CO_2 reaction. Corrected for effect of changing buffer concentration on physical solubility of CO_2.

throughout its *whole* course, as the phosphate concentration is increased; whereas if the phosphate did no more than just "instantaneously" remove the H^+ ions formed by the reaction $H_2CO_3 \rightarrow H^+ + HCO_3^-$, the slopes of curves B, C, D should be the *same* in their early stages, and the increased buffer concentration should exercise no effect until the latter part of the uptake, when the

velocity of the back reactions becomes significant, i.e. a family of curves like those shown in Fig. 3 should have been obtained.

Clearly there is, in addition, some direct effect of the phosphate upon the reaction, as is indeed shown quantitatively by equation (8) which fits the curves plotted in Fig. 2, i.e.

$$-\frac{d\,[CO_2]}{dt} = v_u\,[CO_2] = k_u\,[CO_2]\,\{1 + l_u\,[HPO_4^=]\}, \qquad \ldots\ldots(8)$$

where v_u is the apparent overall velocity constant for CO_2 uptake,

k_u is the true velocity constant of the reaction $CO_2 + H_2O \rightarrow H_2CO_3$,

l_u is a catalytic coefficient.

The numerical values of v_u in Tables I and III have been calculated by the method of Brinkman *et al.* [1933], but a correction has also been inserted for the rate of the back reaction, which in the case of phosphate amounts to 2–20 % and in the case of cacodylate to even higher figures. The allowance for the back reaction was made on usual principles and since it requires a knowledge of the end point of the reaction, this was determined either by continuing the experiment for 30 min. or preferably by a separate experiment in which a suitable amount of carbonic anhydrase was added to the mixture, so that the end point was reached in 5 min.

The time intervals over which v_u has been calculated have usually been 2 min. each, and in any given experiment at least four such intervals have been chosen between 1·5 and 8·0 min., v_u as given in Tables 1 and III thus being the average of four or more values. The individual figures in any one experiment usually agree with the mean to within 10 %.

A rougher, but less laborious, mode of calculation was used for the other data, which are mostly of a preliminary nature. This consisted in measuring the time t for the CO_2 pressure to drop through one or more small specified ranges in the early part of the slow phase of CO_2 uptake. Clearly $v_u = 1/t \times$ a constant, which is obtained from the curve of a solution of known v_u (determined as in Table I).
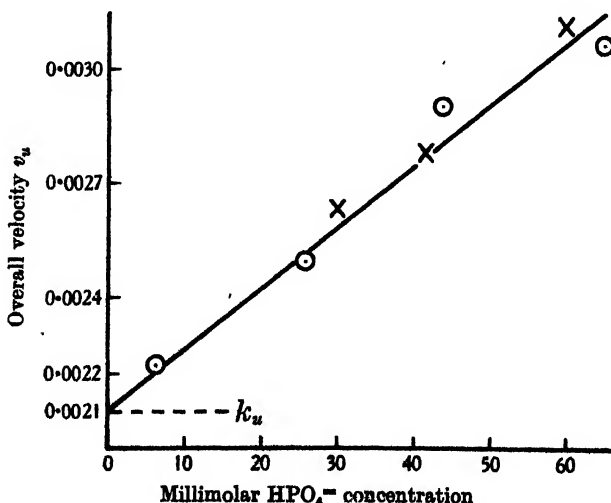
Fig. 4. Effect of $[HPO_4^=]$ on velocity of CO_2 uptake. $\odot$ our experiments; $\times$ calculated from data of Brinkman *et al.* [1933].

Fig. 4 shows that there is a linear relation between v_u and $[HPO_4^=]$, at a ratio of $[HPO_4^=] : [H_2PO_4^-]$ of 2 : 1, as equation (8) requires. The corresponding

value of l_u, namely 8, together with l_u values for ratios ranging from 8 : 1 to 1 : 1 are given in Table I. The l_u values agree with one another within experimental error, thus demonstrating the validity of equation (8).

Table I. *Effects of phosphate and cacodylate on CO_2 uptake rate*

$[HPO_4^-]:[H_2PO_4^-]$	Conc. range (total phosphate, M)	l_u
8·0	0·01–0·20	7·7
3·2	0·01–0·20	8·0
2·0	0·01–0·10	7·7
1·0	0·01–0·10	8·5
Mean		8·0

$[Cac^-]:[HCac]$	Conc. range (total cacodylate, M)	l_u
7·0	0·02–0·20	8·5
4·0	0·02–0·20	9·4
Mean		9·0

A $[HPO_4^-]:[H_2PO_4^-]$ ratio of 1 : 1 was the lowest that could be used, for below this the CO_2 taken up becomes so small and the back reaction increases so fast that accurate determinations are not feasible. For the same reasons it was impossible to reduce the $[Cac^-]:[HCac]$ ratio below 4 : 1, for the pK of cacodylic acid is about 0·8 pH below the pK_2 of phosphoric acid, and even at 4 : 1 the back reaction corrections amount to as much as 30 %, though in spite of this the v_u values over different intervals in any one experiment tally satisfactorily.

Above a total buffer concentration of 0·2 M, the physical solubility of CO_2 is appreciably depressed and the interpretation of the results thus becomes uncertain: in rough experiments, however, up to M it was found that v_u continued to increase nearly linearly with $[HPO_4^-]$. In the case of cacodylate v_u increased rather faster than $[Cac^-]$: this may be just an ordinary deviation from the law of mass action in strong solution, but it might also be due to some change in the state of the cacodylate at higher concentrations, such as the formation of a polymeride which is catalytically more active. A similar deviation occurs with chromate buffer solutions, in which there is known to be an equilibrium between the hydrochromate ion, $HCrO_4^-$, and the dichromate ion, $Cr_2O_7^-$, the amount of the latter increasing rapidly as the total chromate concentration is raised.

It was of interest to see whether equation (8) could also be applied to results published by earlier authors before the effect of the buffer was suspected. Adequate data are available in two such papers, and l_u values calculated therefrom are given in Table II. The excellent agreement with the present findings is a strong confirmation of the validity of our conclusions. Table II further shows that equation (8) is valid over a wide range of CO_2 concentration, namely 0·001 M (present paper) to 0·03 M (Faurholt).

Table II. *Values of l_u calculated from data in the literature*

Source	Buffer	CO_2, M	l_u	l_u from Table I
Faurholt [1925, 1, Table IV]	Cacodylate	0·03	9·5	9·0
Faurholt [1925, 1, Table VI]	Phosphate	0·018	8·4	8·0
Brinkman *et al.* [1933, Table III]	Phosphate	0·002	8·3	8·0

Extrapolation of the data, summarized in Table I (or in Table III [Brinkman *et al.* 1933]) to zero $[HPO_4^-]$ gives us for the first time an accurate value for k_u,

the true velocity constant of the reaction $CO_2 + H_2O \rightarrow H_2CO_3$. The mean value of k_u is 0·0021, i.e. about 25 % lower than the hitherto accepted value of 0·0027 [Table I, Roughton, 1935, 1], which is, of course, wrong, for in the calculations on which it was based no account was taken of the direct catalytic effect of the buffer.

Arguments against the effect being due to neutral salt action. It will be convenient now to exclude the possibility that the effect of the buffer may be accounted for by some kind of "neutral salt action" or ionic activity factor. This we do on the following grounds.

(a) The observed effects are larger than expected, and indeed are appreciable at concentrations, e.g. 0·01 M, much lower than those at which typical neutral salt action manifests itself. This holds not only for phosphate and cacodylate, but more so for the very active substances, such as selenite, tellurate and sulphite, to be dealt with later.

(b) Table III shows that addition of high concentrations of typical neutral salts, such as KCl and NaNO$_3$, to 0·04 M phosphate buffer (1 : 1), only increases the CO$_2$ uptake rate to a relatively slight extent, the l_u values for these salts being at most only one-sixteenth that of phosphate.

Table III. *Effect of neutral salts on the rate of CO_2 uptake by 0·04 M phosphate*

Salt	l_u
$(NH_4)_2SO_4$	0·2
$NaNO_3$	<0·05
$NaNO_2$	0
KCl	0·3
NH_4Cl	<0·05
KI	0·07
KCNS	0·5
$K_4Fe(CN)_6$	0·4

CO_2 output experiments

If the action of the buffer (or some accompanying impurity) is purely catalytic, then the reverse reaction $H_2CO_3 \rightarrow CO_2 + H_2O$ should be equally affected. On the face of it, this should be easy to test by measuring the rate of CO$_2$ output from mixtures of bicarbonate with varying strengths of buffer. Unfortunately, however, variation of the buffer strength will also affect the activities of the ions in solution, and may thus independently alter the rate of CO$_2$ output, which is directly proportional to the product of the H$^+$ and HCO$_3^-$ activities. The exact nature of the possible "activity effects" is shown by the following treatment.

The rate of CO$_2$ output $= \dfrac{d[CO_2]}{dt} = v_o[H_2CO_3] = \dfrac{v_o a_H f_{HCO_3}[HCO_3^-]}{K_1}$, ...(9)

where v_o = overall output velocity constant,

a_H = hydrogen ion activity,

f_{HCO_3} = activity coefficient of HCO$_3^-$,

K_1 = true first ionization constant of H$_2$CO$_3$.

Now $a_H = \dfrac{K_2 f_{H_2PO_4}[H_2PO_4^-]}{f_{HPO_4}[HPO_4^-]}$, ...(10)

where $f_{H_2PO_4}$, f_{HPO_4} = activity coefficients of H$_2$PO$_4^-$ and HPO$_4^-$ respectively,

K_2 = second ionization constant of H$_3$PO$_4$.

Therefore $\dfrac{d[CO_2]}{dt} = \dfrac{v_o K_2 f_{HCO_3} f_{H_2PO_4}[H_2PO_4^-]}{K_1 f_{HPO_4}[HPO_4^-]}[HCO_3^-]$.   ......(11)

The activity coefficients of divalent ions, such as HPO$_4$$^-$, are much more affected, and in a more specific manner, than those of univalent ions, by changes in ionic strength, and there is no reason to suppose that the fraction $f_{HCO_3} \times f_{H_2PO_4}/f_{HPO_4}$ should remain constant as the phosphate concentration is reduced by dilution with water. But if the buffer is, instead, diluted with a mixture of K$_2$SO$_4$ and KCl, of the same total molarity as the highest concentration of buffer and with the same molar proportions of SO$_4$$^-$ to Cl$^-$ as of HPO$_4$$^-$ to H$_2$PO$_4$$^-$ in the buffer, this difficulty should be practically overcome: for Landolt & Bornstein's Tables show that the activity coefficients of K$_2$SO$_4$ and Na$_2$SO$_4$ agree to within 0·01 with the activity coefficient of Na$_2$HPO$_4$ in pure solutions of the respective salts at the same concentration (up to 0·1 M), and it is therefore reasonable to suppose that in phosphate + sulphate mixtures the activity coefficients would be the same as in pure phosphate buffers of the same total molarity. A similar argument is applied to the Cl$^-$ and H$_2$PO$_4$$^-$ ions.

All solutions, except bicarbonate, were freed from CO$_2$ by repeated evacuation and shaking. To compensate for the carbonate content of the bicarbonate solution, an equivalent amount of HCl was placed in the other solution used in the double compartment boat.

Fig. 5. Effect of [HPO$_4$$^-$] on velocity of CO$_2$ output.
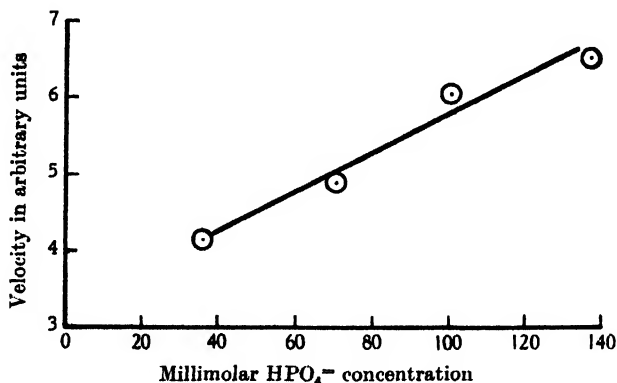
The methods of calculation were similar to those used in the CO$_2$ uptake work. Fig. 5 shows that there is again a linear relation between [HPO$_4$$^-$] and v_o/K_1 and we may therefore write

$$\frac{v_o}{K_1} = \frac{k_o}{K_1}\{1 + l_o [\text{HPO}_4^-]\}, \qquad \qquad \ldots\ldots(12)$$

where k_o is the true velocity constant of the reaction H$_2$CO$_3 \rightarrow$ CO$_2$ + H$_2$O, and l_o is the catalytic coefficient for CO$_2$ output corresponding to l_u for CO$_2$ uptake. Table IV gives the values of l_o for phosphate + sulphate + chloride mixtures and also for cacodylate + chloride ± acetate mixtures. It will be noted that in the case of both buffers l_o is independent of wide variations in pH and that the l_o values agree, within experimental error, not only with the l_u values of Table I but also with the l_u values given in Table IV, which were specially determined in buffer salt mixtures of the same composition as used in the output experiments. This last result proves that phosphate and cacodylate are both true catalysts of the reaction CO$_2$ + H$_2$O $\rightleftharpoons$ H$_2$CO$_3$.

The assumption that the ionic dissociation and reformation of H$_2$CO$_3$ are too fast to limit the rates of uptake or output of CO$_2$, even when the buffer

Table IV. *Effects of phosphate and cacodylate on CO_2 output rate*

	$[HPO_4^{-}] : [H_2PO_4^{-}]$	l_o	l_u
Phosphate $+ SO_4^{-} + Cl^{-}$	3 : 1	8·5	8·9
Phosphate $+ SO_4^{-} + Cl^{-}$	1 : 3	8·6	—

	$[Cac^{-}] : [HCac]$	l_o	l_u
Cacodylate $+ Cl^{-}$	7 : 1	—	8·5
Cacodylate $+ Cl^{-}$	2 : 1	9·2	—
Cacodylate $+ Cl^{-} +$ acetate	1 : 2	8·9	—

concentration is below $M/100$, perhaps requires some further words. Ionic reactions of this kind are generally assumed to be "instantaneous". A minimum value for their rate is given by rapid reaction velocity experiments such as those of Roughton [1930] which have shown that in presence of $M/10$ buffer the ionization of weak acids similar to H_2CO_3 has a half-period of less than 0·0003 sec. —probably far less. Whether decrease of buffer concentration would increase the half-period appreciably is very doubtful, since the ionization in complete absence of buffer, i.e. $H_2CO_3 + H_2O \rightleftharpoons HCO_3^{-} + H_3O^{+}$, is probably *per se* extremely fast. But, even if the rapid ionization of H_2CO_3 in buffer solutions were entirely due to a direct reaction with the buffer anion, i.e. $H_2CO_3 + A^{-} \rightleftharpoons HCO_3^{-} + AH$, a decrease of buffer concentration from $M/10$ to $M/100$ could not raise the upper limit for the half-period to more than $10 \times 0·0003$ sec. $= 0·003$ sec. Now calculation shows that, for the overall rate of CO_2 uptake in $M/100$ buffer solution to be retarded appreciably by the ionization of H_2CO_3, the half-period for the latter must be $> 0·01$ sec. The action of the buffers cannot therefore be due to any effect on the ionic reaction $H_2CO_3 \rightleftharpoons H^{+} + HCO_3^{-}$, but must be due entirely to their effect on the molecular reactions of CO_2 with water. Of several further points in favour of this we need only mention that the numerical values of k_u and k_o/K_1, as found by extrapolating to zero buffer concentration the respective rates of CO_2 uptake and output, check satisfactorily in two independent ways with the requirements of the law of mass action when applied to the molecular reaction $CO_2 + H_2O \rightleftharpoons H_2CO_3$. (Details will be given in a later paper by one of us.) If the rate of ionization of H_2CO_3 were of any limiting influence at low buffer concentrations no such checks would be obtained.

Additive test. An experimental comparison of the rates of CO_2 output from a mixture containing

(a) 0·017 M $NaHCO_3 + 0·075$ M $K_2HPO_4 + 0·025$ M $KH_2PO_4 + 0·1$ M KCac, with

(b) 0·017 M $NaHCO_3 + 0·075$ M $K_2HPO_4 + 0·025$ M $KH_2PO_4 + 0·1$ M Na acetate,

showed that the rate of (a) was 1·52 times that of (b). This demonstrates that the catalytic effects of the phosphate and cacodylate ions are additive, for on this basis we should have

$$\frac{\text{rate of } (a)}{\text{rate of } (b)} = \frac{1 + (8·55)\,(0·075) + (9·05)\,(0·1)}{1 + (8·55)\,(0·075) + (0·6)\,(0·1)} = 1·49.$$

In this fraction 8·55, 9·05 and 0·6 are the respective l_o values of HPO_4^{-}, Cac^{-} and CH_3COO^{-} (Table IV).

The observed and calculated ratios agree much better than might have been expected from the size of the experimental errors.

Evidence that the catalytic action of the buffers is not due to an accompanying impurity. An impurity present in the salts and/or alkalis used in making the buffer solutions might be responsible for the catalytic effects observed.

The alkalis should first be considered, for they are used both by the manufacturer in preparing salts, such as KH_2PO_4, and by ourselves in making up the buffers. Two tests eliminate this possibility. (*a*) The effects are the same whether NaOH or KOH is used. (*b*) No increased effect is found when the buffer is treated with additional alkali if the latter is afterwards neutralized with an equivalent amount of HCl. It will be recalled that Cl^- itself does not inhibit— see Table III.

As regards the phosphate, we find exactly the same amount of catalysis whether all the phosphate solution is made up from KH_2PO_4 (Kahlbaum puriss. or AnalaR) or from $Na_2HPO_4, 12H_2O$ (Kahlbaum puriss. or AnalaR). It seems unlikely that all these reagents would contain just the same amount of catalytic impurity. Furthermore since the effect is always proportional to the $[HPO_4^-]$ or $[Cac^-]$, the impurity would have to have the same pH-activity curve as both phosphate and cacodylate, the pK of which differ by 0·8. This is impossible if the impurities in the two cases are the same, whereas if there are two different impurities it would be a remarkable coincidence that their respective pH-activity curves should both happen to be identical with the ionization curves of the corresponding buffers.

Addition of sufficient carbonic anhydrase to double the rate of CO_2 uptake by $M/50$ phosphate, is also found to double the rate of CO_2 uptake by $M/5$ phosphate which, in absence of enzyme, it will be remembered, is nearly twice the rate of uptake by $M/50$ phosphate. The catalytic actions of carbonic anhydrase and phosphate thus appear to multiply one another instead of being merely additive, as would be expected if the phosphate catalysis were due to traces of an impurity. A possible mechanism for the multiplicative effect is put forward later.

That the effect can be due to metallic impurities is very unlikely because:

(*a*) Certain metals, notably Fe, are precipitated by phosphate at pH 8·0, yet the supernatant fluid from phosphate solutions which had been kept a long time showed the same effect as freshly prepared solutions.

(*b*) No increased effect was observed on adding to 0·04 M phosphate, pH 7·1, various possible impurities in traces, including many cations, e.g. Li^+, Cu^+, Cu^{++}, Ca^{++}, Sr^{++}, Ba^{++}, Mg^{++}, Hg^{++}, Pb^{++}, Fe^{++}, Fe^{+++}, Sb, La, U, Sn. The following cations had no (or very slight) additive effect on the rate of CO_2 uptake by cacodylate buffer, pH 6·8: Ca^{++} (0·2 M), Ba^{++} (0·2 M), Sr^{++} (0·2 M), Th^{++++} (0·001 M), La^{+++} (0·0005 M). At 0·0006 M, however, La^{+++} had a marked accelerating effect, but at this concentration a precipitate developed in the boat. 0·012 M Cu^{++} and 0·1 M Rb^+ in β-glycerophosphate, pH 6·8, had no effect.

(*c*) The rate of CO_2 uptake by phosphate, pH 7·1, is unaffected by adding 0·08 M cyanide.

This varied evidence, taken as a whole, makes it safe for the present to attribute the catalytic action in the main, if not entirely, to the more electronegative constituent of the buffer itself.

Further experiments on the nature of the catalysis

The natural question next arises as to whether this effect is limited to the cacodylate, the secondary phosphate and the borate ions (Faurholt's work) or whether it is shown by the more negative constituent of oxy-acid buffers in general. We have tested the matter by CO_2 uptake experiments on a wide range of buffers, either in pure solution or when mixed with phosphate buffer, in which case their additive effect, if any, on the rate of uptake by phosphate has been worked out. At $pH > 9·0$ results become less easy to interpret owing to the

appreciable intervention of the $CO_2 + OH^- \rightleftharpoons HCO_3^-$ reaction, and we have therefore, for the present, restricted ourselves in the main to buffers with acid constituents of pK below 9·0. Our object being to make a broad preliminary survey of the whole field, we have in most cases contented ourselves with the rougher method of calculating l_u described above, and we have neglected any complications which may arise from the possible effects upon the solubility of CO_2 of certain of the substances which were used in high concentration: for these reasons we do not claim the same exactitude for these results as for those given in Tables I and IV, though we do believe that their order of magnitude is correct. The values of l_u, in terms of the more negative constituent of the buffer, are

Table V. *Effect of various oxy-acid buffers on rate of CO_2 uptake*

Buffer	pK at $0°$	[ion] : [acid]	[ion]+[acid],* M	l_u
I. Phosphate	pK_2 7·1	—	—	8·0
Phosphite	pK_2 6·7	2·0	0·20	6
Pyrophosphate	—	—	—	10–50
α-Glycerophosphate	6·4	10·0	0·40	4·5
β-Glycerophosphate	6·3	3·0	0·25	3·0
Phosphoglycerate	6·0	18·0	0·15	2·5
Hexosediphosphate	6·3	9·0	0·23	8·5
Cacodylate	6·3	—	—	9·0
Arsenate	pK_2 6·8	2·0	1·20	6·0
II. Maleate	pK_2 6·1	8·0	0·95	2·0
			1·00	1·6†
Citrate	pK_3 5·4	c. 20·0	2·00	1·5†
Veronal	8·0	3·0	0·18	8·0
III. Chromate	pK_2 6·4	3·0	0·1	c. 50
Borate	9·5	0·016	1·45	c. 150†
IV. Sulphite	pK_2 7·0	3·0	0·1	900
Selenite	pK_2 8·0	0·3	0·02	1700
			0·0036	>2000†
Tellurate	pK_1 7·8	1·0	0·05	600
V. Formate	3·8	—	1·0	0·5†
Acetate	4·7	—	1·0	0·6†
Phthalate	pK_2 5·3	—	0·8	0·8†
Oxalate	pK_2 4·1	—	0·6	1·4†

* Highest total concentration used. † Measured in 0·04 M phosphate buffer.

given in Table V which shows clearly that the effect sought for is very widespread, being considerable with all oxy-acids of $pK > 6$, and in general increasing with pK. For convenience in discussion Table V has been divided into the following sections.

Group I consists of buffers closely related in structure to phosphate and cacodylate, and with pK in the range 6·0–7·0. Phosphite, with a pK_2 rather lower than the pK_2 of phosphate, has an l_u value also slightly less (i.e. 6·0 compared with 8·0). The organic phosphates with still lower pK have their l_u values correspondingly lower, except hexosediphosphate. But the molecule of this substance has two phosphate groups, each with pK at 6·3. Hence for calculating l_u, a M solution of hexosediphosphate should from the present point of view correspond to a 2·0 M solution of the ion. On this basis, l_u comes to be of the same order as for the other organic P compounds. Pyrophosphate shows considerable catalytic effect, but it is present both in the trebly and quadruply ionized forms under the pH conditions studied, and the respective catalytic contributions of these two forms have not yet been worked out.

Group II. Two organic acids, maleate and citrate, with pK near the lower limit at which activity is shown, have rather small l_u values. Veronal with a pK of 8·0 shows, as would be expected, a higher l_u value.

Group III. The two inorganic buffers chromate and borate have l_u values about 10 times greater than those in group I. In the case of chromate there is, as already mentioned, some uncertainty owing to the presence in solution of appreciable amounts of $Cr_2O_7^-$, besides the $HCrO_4^-$ and CrO_4^- ions.

Faurholt [1925, 1] commented on the anomalous results found by him with borate even at pH 8·0, where complications arising from the $CO_2 + OH^- \rightarrow HCO_3^-$ reaction should only be slight. Inspection of his figures shows that the discrepancies to which he refers could be explained if we assign an l_u value to the borate ion of the order of 100–200—a reasonable figure in view of the pK of boric acid, i.e. 9·5. In borate-phosphate mixture we find an l_u value of about 150, thus confirming this suggestion.

Group IV. Sulphite, selenite and tellurate,[1] showed the greatest effects of any so far tested, l_u being in the neighbourhood of 1000. Careful controls showed that the results were not due to autoxidation. Their activity, being so much greater than that of other substances of similar pK, must be due to their special chemical constitution: in this connexion it is of interest to find this high activity associated with three elements of the same group (VI) of the Periodic Table. A further point of interest about the action of selenite will be discussed later.

The effects of chromate and selenite on CO_2 output were also investigated. The l_0 values in the two cases were found to be of the same order as the l_u values.

Group V contains some typical carboxylic acids with $pK < 5·0$. Their l_u values, with the doubtful exception of oxalate, are all distinctly below 1·0. It thus appears that as the strength of the acid rises a range is reached (pK 6·0–5·0) in which the catalytic effect of the acid anion tends to fade out.

The effect of the bicarbonate ion

The l_u value for the HCO_3^- ion is not included in Table V, since it cannot be obtained either by the methods so far used in this paper or from data given in previous papers. It is, however, important to measure it, both from the point of view of the physico-chemical mechanism of the $CO_2 + H_2O \rightleftharpoons H_2CO_3$ reaction and also of practical applications to biological problems. Since the true first pK of H_2CO_3 is about 3·7 the l_u value for HCO_3^- would not, from Table V, be expected to exceed 1·0. The following modification of the usual technique shows that, in point of fact, the l_u value must be less than 0·5 and may be zero.

A 0·2 M $NaHCO_3$ in 0·004 M Na_2CO_3 mixture was prepared by dissolving the requisite weights of the two salts in recently boiled distilled water with minimal contact with air so as to reduce CO_2 exchange. 5 ml. of this solution were placed in the boat and shaken at 0° with the standard CO_2 pressure (in the usual way) for 90 min., i.e. until equilibrium was practically reached. The $[CO_3^-]$ was found to have increased to 0·005 M, and the $[HCO_3^-]$ to have dropped correspondingly. The pH of the solution was calculated to be 8·6. After 1 min. pause for drainage, a second vol. of CO_2 equal to the first was introduced into the boat in the usual way, and an ordinary experiment then carried out to completion. Still more CO_3^- was formed and there was a further slight fall in pH. The overall velocity constant of CO_2 uptake was then calculated from the time course of the CO_2 uptake during the second shaking though far larger corrections were necessary than usual for (a) the velocity of the back reaction (50–60 %) and (b) the velocity

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[1] Prepared from B.D.H. telluric acid. Two samples from other sources were unsatisfactory as they gave heavy precipitates when partially neutralized by alkali.

of the $CO_2 + OH^- \rightarrow HCO_3^-$ reaction which at pH 8·6 is about 30 % of the velocity of the $CO_2 + H_2O \rightarrow H_2CO_3$ reaction. Fortunately, these two large corrections are opposite in sign, and in consequence the final value of the overall velocity constant should be accurate to about ± 10 %. The average of four calculated values came out to 0·00208, in very close agreement with the value, 0·0021, given above for k_u, the true velocity constant of the reaction $CO_2 + H_2O \rightarrow H_2CO_3$. The joint catalytic effects of the HCO_3^- and CO_3^- ions in this experiment thus cannot at most have exceeded 10 %, which means that l_u for HCO_3^- ion alone ≯ 0·5—a negligible value for practical purposes in work at $[HCO_3^-] < 0.05$ M.

The experiment also gives an upper limit of about 25 for the l_u value of the CO_3^- ion. Recent experiments at higher $[CO_3^-]$ suggest that the limit is almost certainly much lower.

II. BASIC NITROGENOUS BUFFERS AND OTHER SUBSTANCES

Basic nitrogenous buffers

We have tested three classes of N-containing bases: (a) compounds with the basic N in a closed ring, e.g. glyoxaline, nicotine, (b) compounds with the basic N in a straight chain, e.g. aniline and (c) mixed compounds containing both cyclic basic N and straight chain basic N, e.g. histidine. The results with class (a) being the most straightforward will first be described.

Cyclic N bases. Four members of the glyoxaline series and three other compounds were tested as regards their effect on CO_2 uptake by the methods described in Section I (see Table VI). Fig. 6 shows a typical pair of results for

Table VI. *Effect of cyclic nitrogenous bases*

Buffer	pK at 0°	[base] : [ion]	l_u	l_o
2:4(or 5)-Dimethylglyoxaline	8·80	0·33	12·5	—
4(or 5)-Methylgloxaline	7·97	2·0	9·5	—
		1·0	11	—
		0·5	9·5	—
		0·25	10	—
Glyoxaline	7·40	1·0	1·5	—
		2·0	—	c. 2
4(or 5)-Hydroxymethylglyoxaline	6·85	4·0	1·6	—
Nicotine	8·5 (8·0 at 25°)	0·5	13	—
Pilocarpine	7·30 (7·0 at 15°)	1·0	4·6	—
		1·0	—	5·0
Pyridine (in phosphate)	5·7 (5·3 at 20°)	50·0	<0·6	—

an equimolar buffer mixture of glyoxaline and glyoxaline hydrochloride at total concentrations of glyoxaline ranging from 0·036 to 0·38 M. It will be noted that the CO_2 uptake during the initial rapid phase is in both cases practically the same and is equal in amount to that expected from the solubility coefficient of CO_2, thus showing that in this case there is no rapid reversible combination of the base with CO_2 to form a compound of a carbamino type. (Contrast the results obtained in Fig. 7 with a typical straight chain N compound.) Nor was there any such indication in the case of any of the other compounds listed in Table VI.

When v_u, the overall velocity constant for CO_2 uptake, is calculated as in Section I and plotted against the concentration of unionized base (i.e. the more negative constituent of the buffer) a straight line is obtained. The slope of the

line shows that the l_u value for glyoxaline is about 1·6. Extrapolation of the line back to zero base concentration gives a value for k_u, the true velocity constant of the reaction $CO_2 + H_2O \rightarrow H_2CO_3$, in close agreement with that already found in Section I.

The l_u values given in Table VI have all been calculated in terms of the more negative constituent: the validity of this procedure was tested thoroughly in the case of 4(or 5)-methylglyoxaline by experiments at four different [N base : [N cation]] ratios ranging from 2·0 to 0·25. It is seen that l_u is constant within experimental error. It may be reasonably assumed that the other compounds in the Table would, on test, yield the same result.

Table VI also shows that there is a correlation between l_u and pK similar to that already found for the oxy-acid buffers in Table V (though in the present case the available range is smaller): thus, for example, the two compounds with $pK > 8·0$, nicotine and 2:4(or 5)-dimethylglyoxaline, each have an l_u of about 12, whereas pyridine with a $pK < 6$ has a barely detectable l_u value. The compounds of intermediate pK have, broadly speaking, intermediate l_u values.

Fig. 6.

Fig. 7.

Fig. 6. Observed course of CO₂ uptake by glyoxaline. A, 0·018 M unionized glyoxaline + 0·018 M glyoxaline ion; B, 0·19 M unionized glyoxaline + 0·19 M glyoxaline ion.

Fig. 7. Observed course of CO₂ uptake by hydroxylamine buffer solutions. A, 0·036 M NH₂OH + 0·012 M NH₃⁺OH; B, 0·085 M NH₂OH + 0·026 M NH₃⁺OH/HPO₄⁻ + 0·011 M; C, 0·033 M H₂PO₄ for comparison.

Evidence for the catalytic nature of the effect was sought for as before, namely by comparing the l_0 values, obtained from CO₂ output experiments at different buffer concentrations, with the l_u values. Unfortunately, owing to experimental error and theoretical uncertainty, satisfactory accuracy can only be attained, if the l_0 value is > 5 and the $pK < 7$ (as is the case both for phosphate and cacodylate buffers). Of the compounds listed in Table VI pilocarpine approaches this ideal most nearly: the l_0 value from experiments over a range of 0·05–0·20 M total pilocarpine concentrations is 5·0, in satisfactory agreement with the l_u value of 4·6. A similar, but much less accurate test showed that the

l_o and l_u values of glyoxaline are of the same order of magnitude. The actual equation for the overall velocity constant of CO_2 output by N base buffers differs slightly from equation (11): it is

$$\frac{d[CO_2]}{dt} = \frac{v_o K_B f_{HCO_3} f_B [B^+]}{[B]} [HCO_3^-], \qquad \ldots\ldots(13)$$

where $[B^+]$, $[B]$ are the concentrations of N cation and base respectively.

K_B is the ionization constant of the base $= [B][H^+]/[B^+]$.

f_B is the activity coefficient of the N cation.

The calculation of l_o in the case of N base buffers is thus much more sensitive to activity coefficient errors than in the case of the oxy-acid buffers, since a product of activity coefficients is involved instead of a quotient of activity coefficients as in equation (11).

Straight chain N bases. Faurholt [1925, 2] has measured the equilibrium constant K_{diss} for the carbamino reaction between CO_2 and the following straight chain amines: NH_3, CH_3NH_2, $(CH_3)_2NH$ and $CHNH_2COO^-$ (glycinate). As a measure of affinity of the bases for CO_2 we may take

$$1/K_{diss} = \frac{[CO_2][B]^2}{[B^+][carb]}, \qquad \ldots\ldots(14)$$

where [carb] is the concentration of carbamino compound formed. The values of $1/K_{diss}$ at $0°$ ranged from 10^3 to 10^6 indicating a very large affinity of these bases for CO_2. This, together with the fact that the pK value at $0°$ in all four cases is > 10, makes it very difficult by the present methods to determine whether these bases have an appreciable l_u value, though Faurholt's work indirectly speaks against such a possibility.

We have, however, tested several straight chain bases of $pK < 9\cdot0$, and, in accordance with Faurholt's views, have found a much lower carbamino affinity for CO_2 so that it has been possible to allow for it fairly well in calculation of l_u. Typical results for hydroxylamine at a $[B]:[B^+]$ ratio of $3:1$ and total concentrations $0\cdot048\,M$ and $0\cdot11\,M$, are shown in Fig. 7.

The CO_2 uptake in the rapid phase is much greater than that taken up in physical solution (see control curve for CO_2 uptake by phosphate, and also Fig. 2) the excess, due to carbamino formation, is, as is to be expected from equation (14), proportional (at constant $[B]:[B^+]$) to the total hydroxylamine concentration. The data of Fig. 7 indicate that $1/K_{diss}$ for hydroxylamine is about 4, i.e. about 1/500 the value for ammonia, thus showing the marked effect of substituting an —OH group both on pK and on the affinity for CO_2.

It will be seen that the rate of CO_2 uptake during the slow phase is less in the $0\cdot048\,M$ hydroxylamine solution than in the phosphate control, and in the $0\cdot11\,M$ hydroxylamine solution less still. This is partly due to the lower pressure of CO_2 remaining in the gas phase and partly due to the fact that the $[HCO_3^-]$ in the hydroxylamine solution is formed not only from CO_2 coming from the gas phase but also from CO_2 dissociating from the carbamino compound, which is maximal at the end of the rapid phase and decreases as the solution becomes more acid [v. Roughton, 1935, 2]. Approximate corrections can be made for both of these effects: the former is obviously proportional to the extent by which p_{CO_2} is lower than the control, whilst the latter can be shown to be roughly equal to the $[carb]/[CO_2]$ in solution at the end of the rapid phase, provided that the [carb] is not too large. The carbamino corrections in the case of Fig. 7 amount to 7 and 20 % respectively and when applied, together with the p_{CO_2} corrections, show that the true rate of HCO_3^- formation in the hydroxylamine

solution is, within experimental error, equal to the basal rate in absence of catalyst, thus showing that hydroxylamine has no appreciable l_u value. Similar results were obtained with the other compounds listed in Table VII.

Table VII. *Straight chain N bases*

Compound	pK	Carbamino formation	l_u
Hydrazine	8·4 at 20°	+ + +	Undetectable
Hydroxylamine	6·2 at 20°	+ +	,,
Aniline	4·5 at 20°	+	,,
Acetamide	c. 0	0	,,
Urea	c. 0	0	,,

+ + + denotes that $1/K_{diss}$ is of same order as $1/K_{diss}$ for NH_3.
+ +   denotes that $1/K_{diss}$ is of the order of 10^{-2} times $1/K_{diss}$ for NH_3.
+   denotes that $1/K_{diss}$ is of the order of 10^{-4} times $1/K_{diss}$ for NH_2.
0   denotes that $1/K_{diss}$ is less than 10^{-6} times $1/K_{diss}$ for NH_3.

Mixed compounds. From the above results we should expect that a compound which possesses both a basic cyclic N and a basic straight chain N should act both as CO₂ carrier and CO₂ catalyst. We have verified this in preliminary experiments on histidine, and two of its derivatives of physiological interest, dissolved in phosphate buffer. The results are shown in Table VIII.

Table VIII. *Effects of histidine and derivatives on CO₂ uptake rate*

Compound	pK^* at 22°		l_u	Carbamino formation
	Glyoxa-line N	Side chain N		
Histidine	6·15	9·3	c. 1·2	+ + +
Carnosine	6·8	9·5	c. 6	+ + +
Anserine	7·0	9·5	c. 3	+ + +

* Values and assignments from Deutsch & Eggleton [1938] who kindly supplied samples of anserine and carnosine.

Other buffers

We have also tested the catalytic effects of the anions of three non-oxy-acid weak acids, namely HCN (pK at 0° c. 10·0), H_2S (pK at 0° c. 7·0) and HF.

Cyanide. The l_u of CN^- was difficult to measure owing to the volatility and high pK of HCN. The following special technique was used. Scheele's HCN solution (4%, B.D.H.) was mixed with an equal volume of water in a burette over mercury, to avoid HCN loss by evaporation. Titration to pH 6·0 gave the mineral acid content of the solution (0·031 N), and further titration to pH c. 12 (Tropoeolin O as indicator) gave the HCN content (0·79 N). 4 ml. of this solution + 0·8 ml. of M/2 KCN were placed in the boat, the latter stoppered at once and the experiment carried out as usual except that the gas pressure was left at 1 atm. instead of being reduced. The standard amount of CO₂ was introduced from a reservoir at 19 cm. Hg positive pressure. The rate of uptake of CO₂ by this cyanide buffer mixture, which contains 0·66 M HCN and 0·06 M CN^-, was found to be about twice that of the basal rate below pH 7·5 in absence of catalyst. Fourfold dilution of the cyanide buffer in a second experiment showed a CO₂ uptake rate about 1·9 times the basal rate. The increase above the basal rate in the two experiments was mainly due to the rate of the CO₂ + OH⁻ reaction, which in a 1 : 11 cyanide-HCN buffer mixture (pH c. 9·0) should be of the order of 80 %[1] of the CO₂ + H₂O → H₂CO₃ basal rate. If the residue of

[1] Exact allowance is difficult owing to uncertainty as to the pK of HCN at 0° and of the size of the appropriate activity corrections.

the increase is attributed to the catalytic effect of the CN^- ion, its l_u value comes out to be roughly 1·0 or less. CN^- therefore does not appear to belong to the catalytic cyclic N family, which at such a high pK should show an $l_u > 10·0$. Nor does it form any carbamino compound, according to the present experiment and the previous ones of Meldrum & Roughton [1933, 2].

Sulphide. The pK_1 of H_2S at 0° is about 7·0 so that a catalytic effect of HS^- might possibly be expected. Unfortunately the high volatility of H_2S leads to appreciable loss of H_2S into the gas phase of the boat from the liquid as the latter becomes more acid during the CO_2 uptake. To minimize this, the $[HS^-]$ was kept down to 0·012 M by dissolving the requisite amount of NaHS (obtained by half-neutralizing Na_2S (A.R.) with HCl) in a buffer mixture containing 0·05 M 2:4(or 5)-dimethylglyoxaline base + 0·05 M dimethylgloxaline cation (pH c. 9·0). Calculation showed that, even so, a correction of 15 % must be inserted. The experiment was done in much the same way as the cyanide experiment and the uptake rate was found to be about 15 % slower than the control uptake rate by the same buffer mixture without added NaHS. Thus when the correction for H_2S volatility is applied the two rates agree within experimental error, showing that the catalytic effect of 0·012 M HS^- is inappreciable. The l_u value of the HS^- cannot therefore exceed 10·0 and may be zero.

The amount of CO_2 taken up during the rapid phase was found to be the same whether HS^- was present or not, thus showing no evidence of any appreciable rapid reversible combination between CO_2 and HS^- analogous to the carbamino reaction.

We have not yet tested any more complicated sulphhydryl acids, such as thiolacetic acid, owing to the higher pK of their –SH group (c. 10·0) and their instability.

Fluoride. The pK of HF is 4·45 at 25°, and the l_u value, calculated from an additive experiment with 0·6 M NaF + phosphate buffer, is 1·1. This value agrees closely with that of the oxy-acids of pK c. 4·0, and is distinctly higher than the range found for neutral salts of strong acids in Table III.

DISCUSSION

The mechanism of the catalysis

In the CO_2 output experiments the additional rate due to the catalysis is proportional to the product of the $[H_2CO_3]$ and the more negative constituent of the buffer, e.g. $[HPO_4^-]$ or $[Cac^-]$, and is independent of the $[HCO_3^-]$ except in so far as the latter conditions the $[H_2CO_3]$. This means that H_2CO_3, not HCO_3^-, is the substrate acted on by the catalyst, i.e. the reaction catalysed must be $CO_2 + H_2O \rightleftharpoons H_2CO_3$ and not $CO_2 + OH^- \rightleftharpoons HCO_3^-$. Preliminary confirmation of this has been obtained by CO_2 uptake experiments between pH 9·0 and 10·0 with two different $[H_2BO_3^-]$: $[H_3BO_3]$ ratios and varying total borate concentration. It was found that the fraction of the overall uptake rate due to the $CO_2 + OH^- \rightarrow HCO_3^-$ rate was practically unaffected by changes in $[H_2BO_3^-]$ although the fraction due to the $CO_2 + H_2O \rightarrow H_2CO_3$ was greatly affected by $[H_2BO_3^-]$, which showed its usual l_u value > 100.

Whilst not excluding the possibility of a chain mechanism we think it more likely that the catalysis can be explained by the intermediate compound formation between CO_2 and H_2CO_3 on the one hand, and the more negative constituent of the buffer on the other.

In the case of the oxy-acid buffers the compounds might be of a "carbonato"

type, analogous to the reversible compounds between CO$_2$ and organic hydroxides studied by Faurholt:

$$\begin{array}{c}O\\[-4pt]\diagdown\\[-4pt]O\diagup\end{array}\!\!C+OX^- \;\rightleftharpoons\; \begin{array}{c}-O\\[-4pt]\diagdown\\[-4pt]O\diagup\end{array}\!\!C-OX\,(+H_2O) \;\rightleftharpoons\; \begin{array}{c}-O\\[-4pt]HO\diagup\end{array}\!\!\!C-OX \;\rightleftharpoons\; \begin{array}{c}O\\[-4pt]\diagdown\\[-4pt]HO\diagup\end{array}\!\!\!C+OX^-$$

We have to suppose that H$_2$O can combine with the CO$_2$ bound to OX$^-$ to form the corresponding H$_2$CO$_3^-$.OX intermediate compound which in turn decomposes to H$_2$CO$_3$ + OX$^-$. We then have the catalytic scheme formulated in the equation. This scheme explains why (a) the reaction CO$_2$ + OH$^-$ $\rightleftharpoons$ HCO$_3^-$ is not catalysed; probably the electrostatic repulsion between HCO$_3^-$ and OX$^-$ hinders the formation of the necessary HCO$_3^-$.OX$^-$ intermediate compound, and (b) the catalytic activity of the oxy-acid ion tends to disappear when the pK of the acid is < 6·0. Table VII shows how the affinity of the straight chain N bases for CO$_2$ declines *pari passu* with the decrease in pK, i.e. decreases in affinity for H$^+$. Similarly we might suppose that the combination of CO$_2$ with the —O$^-$ in weak acid anions persists up to a certain point as the strength of the acid increases but finally tends to become negligible. On this view the lack of catalysis is due to failure to form the necessary intermediate compound.

In the case of the cyclic nitrogenous base buffers the intermediate compound with CO$_2$ might be of a carbamino type:

$$\begin{array}{c}O\\[-4pt]\diagdown\\[-4pt]O\diagup\end{array}\!\!C+HNY \;\rightleftharpoons\; \begin{array}{c}O\\[-4pt]\diagdown\\[-4pt]HO\diagup\end{array}\!\!\!C-NY\,(+HO_2) \;\rightleftharpoons\; \begin{array}{c}HO\\[-4pt]HO\diagup\end{array}\!\!\!CNY \;\rightleftharpoons\; \begin{array}{c}O\\[-4pt]\diagdown\\[-4pt]HO\diagup\end{array}\!\!\!C+HNY$$

With straight chain N compounds no reaction occurs between water and the carbamino compound of the N bases, but in the cyclic N compounds the postulated carbamino compound might, for some reason connected with the chemistry of the ring, tend to hydrate, forming the unstable intermediate which breaks down to H$_2$CO$_3$ and the free N base. The catalytic scheme is thus complete. Although at present we have no direct evidence in favour of these hypotheses, we have found them very useful working guides, and therefore have felt justified in mentioning them.

Additive effects

We have already alluded to the fact that the catalytic effects of HPO$_4^-$ and Cac$^-$, when jointly present, are additive and the same is also true of the following pairs—maleate + phosphate, chromate + phosphate and probably also borate + phosphate. Carbonic anhydrase and phosphate, on the other hand, tend to multiply the effects of each other. It was therefore of special interest to see whether our most active inorganic catalyst, selenite, is additive or multiplicative. It is difficult, owing to ionic activity uncertainties, to make such experiments fully satisfactory from a theoretical standpoint, but some preliminary results with selenite + phosphate, and selenite + chromate mixtures do suggest that in both cases selenite multiplies the effect of the weaker catalytic ion. Selenite may thus turn out to be a weak inorganic analogue of carbonic anhydrase, in which case a further detailed study of its catalytic mechanism would be well worth while.

As to the mechanism of the multiplicative effect it is possible that selenite and/or carbonic anhydrase may catalyse the formation of the intermediate compound between CO$_2$ and the more weakly catalytic anions, which we have supposed to be a preliminary to the catalysis and which may not itself proceed very rapidly. Various other explanations are however possible.

The effect of pH on carbonic anhydrase

As already hinted in the introduction, the original aim of the present research was to determine the effect of pH upon the activity of carbonic anhydrase.

Two difficulties were already obvious at the start, namely (a) in the pH range above 8·0, where CO_2 reacts in two ways (see equations (1) and (2)) there is the problem of sorting out the respective effects of the enzyme on these two different processes, and (b) the Michaelis constant of the enzyme is very high, and hence it is impracticable to work at substrate concentrations sufficient to saturate the enzyme—a prerequisite in pH-activity work. The results described in this paper, though at first sight adding yet a further complication, may on further investigation prove to simplify the study of the effect of pH on carbonic anhydrase. Thus in regard to (a) preliminary experiments suggest that carbonic anhydrase, like phosphate and other buffers, only catalyses the $CO_2 + H_2O \rightleftharpoons H_2CO_3$, and does not affect the $CO_2 + OH^- \rightleftharpoons HCO_3^-$. In regard to (b), it is already known from unpublished observations of the writers and others, that the activity of carbonic anhydrase increases markedly over the pH range 6·0–8·0. If it turns out that the activity of the enzyme in promoting CO_2 uptake at constant CO_2 pressure when plotted against pH gives a curve resembling the ionization curve of a weak acid, a result exactly analogous to the results with phosphate etc. would be obtained, and it might by further analogy be fair to infer therefrom the actual pK of the active group of the enzyme, even though the enzyme had not been saturated with substrate in the experiments. All this is perhaps too much to hope for, but at all events we have some valuable new pointers as to the investigation of the mode of action of carbonic anhydrase.

The catalytic effects herein described occur in many physiological buffers containing CO_2, and should therefore be borne in mind as possible factors in diverse physiological and biochemical processes and experiments. In particular, they may be of interest in bone equilibria, wherein carbonato-phosphate reactions of a type somewhat similar to that postulated in our catalytic schemes may take place, especially in regions of high local phosphate concentration.

SUMMARY

1. Detailed improvements have been made in the manometric technique for measuring the rate of CO_2 uptake by solutions.

2. When CO_2 is taken up by buffer solutions the buffer substance itself has some direct effect on the reaction besides "instantaneous" removal of H^+ concerned in the ionization of H_2CO_3 to $H^+ + HCO_3^-$. Experimental proof is offered that the effect cannot be due to impurities in the solutions or to "neutral salt" action, but is proportional to the concentration of the more negative constituent of the buffer. Thus in the case of phosphate buffer, the overall velocity constant, v_u, is given by

$$v_u = 0 \cdot 0021 \, [CO_2] \, \{1 + l_u \, [HPO_4^-]\},$$

l_u, the catalytic coefficient, being 8, and for cacodylate 9. The figure 0·0021 represents the true velocity constant of the reaction $CO_2 + H_2O \rightarrow H_2CO_3$ and is about 25 % lower than the erroneous value previously accepted.

3. All other oxy-acids, so far tested, which buffer in the pH range 6–9 show similar effects, whereas salts of stronger acids have much smaller effects ($l_u < 1 \cdot 5$). The effects with sulphite, selenite and tellurate are much larger (l_u of order of 1000).

4. Straight chain N bases, e.g. NH_2OH, show no appreciable catalytic activity, though they combine readily with CO_2 to form carbamino compounds. Cyclic N bases, e.g. glyoxaline, on the other hand do not form carbamino compounds but do act catalytically. The effect is proportional again to the concentration of the more negative constituent—in this case the unionized N base. Values for l_u of the order of 10 were found for several cyclic bases of $pK > 7 \cdot 0$. With weaker bases the effect tends to disappear. Mixed compounds, e.g. histidine, show both catalytic action and carbamino formation.

5. Similar effects are observed on the rate of *output* of CO_2 from bicarbonate solution suddenly mixed with buffer, l_0 (output) for the oxy-acid buffers phosphate, cacodylate, chromate, selenite, and for the N base buffers pilocarpine and glyoxaline being equal to l_u. The effect must therefore be a catalysis.

6. It is shown that the buffers only catalyse the $CO_2 + H_2O \rightleftharpoons H_2CO_3$ reaction and not the $CO_2 + OH^- \rightleftharpoons HCO_3^-$ reaction. A mechanism is suggested in which CO_2 (or H_2CO_3) combines reversibly with the more negative constituent of the buffer.

7. The effects of phosphate and cacodylate are additive, but of the enzyme and phosphate are probably multiplicative.

8. Some biochemical implications of these results are discussed. They must be allowed for in work involving velocities of CO_2 reactions. Selenite is suggested as a possible inorganic model of carbonic anhydrase.

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CCLXVII. BLOOD SUGAR AND BLOOD CHLORIDE CHANGES IN THYROIDECTOMIZED RATS FOLLOWING EXPOSURE TO VARIOUS DAMAGING AGENTS

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PREVIOUSLY described experiments have shown that exposure of animals to non-specific damaging agents, such as toxic doses of drugs, surgical shock, excessive muscular exercise, cold etc., elicits a syndrome with characteristic changes in various organs and in blood chemistry. These changes are largely independent of the specific nature of the damaging agent to which the organism is exposed. However, if an animal is subjected beforehand to a damaging stimulus for a few days, it will no longer respond in this manner to further treatment with the same agent. It was concluded, therefore, that the syndrome represents the somatic expression of the "alarm" of the organism when first confronted with a stimulus to which it is quantitatively or qualitatively not adapted. For this reason, the syndrome has been termed the "alarm reaction" [Selye, 1937, 1, 2; 1938, 1; Harlow & Selye, 1937].

Among the blood chemical changes which characterize the alarm reaction, an initial increase followed by a marked decrease in blood sugar was found to be very constant. With continued treatment this hypoglycaemia disappears within a few days and after resistance to the stimulus is acquired, further treatment will actually increase the blood sugar above the normal level. The blood chlorides show a marked decrease during the alarm reaction and this is followed by an increase after adaptation [Selye, 1938, 2, 3].

It was observed furthermore that removal of endocrine glands which influence the adaptability of the organism may considerably alter this chemical response in the blood. Thus adrenalectomized [Selye, 1938, 4] or hypophysectomized [Selye & Foglia, 1938] animals show no initial hyperglycaemia but a very pronounced hypoglycaemia when exposed to agents capable of eliciting an alarm reaction. The hypoglycaemia is usually most marked in animals which are particularly severely damaged, hence it was not surprising to note an especially marked decrease in the blood sugar in hypophysectomized or adrenalectomized animals, since animals deprived of these glands become unusually sensitive to any damaging agent. It was very unexpected, however, to find in the course of these experiments that removal of the thyroid has an exactly opposite effect, inasmuch as it exaggerates the hyperglycaemic phase of the response. It was noted furthermore that in a group of thyroidectomized rats, the most severely damaged individuals are usually the ones which show the most pronounced hyperglycaemia. In spite of this inverse blood sugar response, the blood chlorides react in the usual manner; in fact, the hypochloraemia is exceptionally pronounced. The purpose of this communication is to report on a series of experiments illustrating these points.

METHODS

The blood sugar determinations were carried out by the Shaffer-Hartmann-Somogyi method and the blood chloride determinations by the Van Slyke method. The chlorides were determined in whole blood specimens, not in plasma, because in some cases it was found that a severe alarm reaction may be accompanied by intravital haemolysis. In every case the animals were fasted 24 hr. prior to the determination so as to avoid any change in blood composition resulting from the ingested food. One animal was used for one determination only, so as to avoid changes resulting from repeated fasting and bleeding.

Female "hooded" rats weighing 95–135 g. were used in all the experiments of this series. The alarm reaction was elicited by exposure to cold, formaldehyde treatment or muscular exercise. The animals treated with formaldehyde received 0·2 ml. of a 4 % solution subcutaneously three times the first day and once the next morning, 23 hr. after the first injection. They were killed one hour after the last injection. Those exposed to cold were placed in an ice-box at +3° for a period of 24 hr. Those forced to perform muscular exercise were placed in drum cages having a diameter of 12 in. and revolving at a speed of 18 r.p.m. They were forced to make three 15 min. runs in the course of the first day, and one the following morning. They were killed 1 hr. after the beginning of this last exercise period. The total length of treatment was 24 hr. in each case, during which period no food was allowed. This length of treatment was chosen because in normal animals the blood sugar curve usually reaches a low point at about 24 hr. after exposure to an alarm reaction-eliciting agent. 8 normal and 8 thyroidectomized rats were exposed to each of the agents and two similar groups of otherwise untreated rats served as controls. The thyroidectomies were performed 9 days before the beginning of treatment. It should be emphasized that in the rat the two internal parathyroids are enclosed in the thyroid tissue and were consequently removed together with the thyroid. Although no signs of tetany were evident in animals thus operated upon, it is possible that ablation of these parathyroids may have influenced the response. Experiments are now in progress in this laboratory in which only the parathyroids are removed. These may throw some light on this aspect of the problem.

RESULTS

Table I summarizes the results of these experiments. The numbers in parentheses represent extreme variations.

Table I

Damaging agent	Blood sugar, mg. per 100 ml.		Blood chlorides, mg. per 100 ml.	
	Normal	Thyroidectomized	Normal	Thyroidectomized
Formaldehyde	76 (70–85)	111 (81–144)	386 (363–421)	356 (339–374)
Exercise	70 (56–80)	78 (65–82)	463 (456–479)	407 (385–410)
Cold	74 (55–80)	91 (70–112)	407 (386–433)	384 (362–395)
Controls	85 (81–92)	70 (60–80)	435 (421–456)	423 (410–433)

It is quite obvious that exposure to the various damaging agents used in this experimental series led to a marked fall in the blood sugar of the normal animals, while in the thyroidectomized rats exposure to these same stimuli not only failed to produce such an effect but actually caused hyperglycaemia. This is particularly interesting in the case of animals exposed to cold or performing muscular exercise, since these stimuli call for increased combustion of carbohydrate

reserves. It should also be emphasized that following a 24 hr. fasting period, the blood sugar of thyroidectomized rats is 15 mg. per 100 ml. below that of the normals, a fact which we have confirmed in the course of many experiments performed in connexion with other problems. In spite of this lower initial value, the blood sugar of the damaged thyroidectomized rat is far above that of the normal. The blood sugar concentration was usually highest in those rats which appeared to be most severely damaged. The blood chlorides of the thyroidectomized rats, on the other hand, decrease even more markedly than those of the normals. A particularly interesting point in this connexion is the behaviour of the blood chlorides in the exercised group. Previous experiments have shown that whilst an alarm reaction produced by any damaging stimulus so far examined causes hypochloraemia, that elicited by excessive muscular exercise results in a pronounced rise in blood chlorides [Selye, 1938, 3]. It was found, however, that the same amount of exercise which increases the blood chlorides in the normal rat causes hypochloraemia in the adrenalectomized animal [Selye, 1938, 4]. A similar change in the response was also observed in the thyroidectomized rats of the present series, as will be seen from the data in Table I; the blood chlorides of the normals rose while those of the thyroidectomized rats fell under the influence of the same amount of muscular exercise.

Since prolonged fasting also elicits typical signs of the alarm reaction (hyperplasia of the adrenal cortex, loss of adrenaline from the medulla, atrophy of the thymus, lymph glands and spleen etc.), it seemed of interest to examine the effect of this stimulus on the blood sugar of thyroidectomized rats. 24 normal and 24 thyroidectomized rats comparable in every respect with those in the

Table II

Fasting period hr.	Blood sugar, mg. per 100 ml.	
	Normal	Thyroidectomized
24	85 (80–91)	70 (61–79)
48	76 (69–81)	94 (83–112)
72	62 (46–69)	65 (55–76)

above-mentioned experiments were used to elucidate this point, one-third of them having been fasted for 24, another third for 48 and the remainder for 72 hr. The results of the blood sugar determinations in these groups are summarized in Table II and Fig. 1

It will be seen, in conformity with what we said above, that after 24 hr. fasting, the blood sugar of the thyroidectomized animal is considerably below that of normal controls fasted for the same length of time. Yet after 48 hr. fasting, when morphological signs of the alarm reaction become evident, the blood sugar rises, not only above that of normal controls fasted for the same period, but even above that of normal animals fasted for 24 hr. only. At the end of 72 hr., however, the blood sugar values of both groups are very low and not significantly different from each other. We do not wish to speculate concerning

Fig. 1.
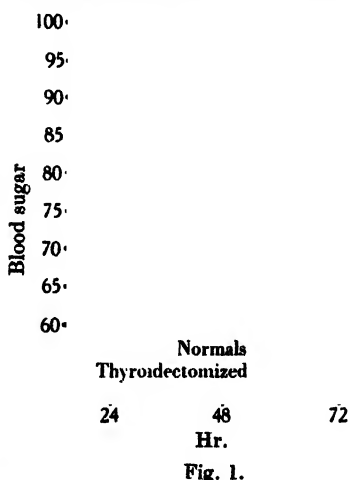
the fundamental disturbances in the carbohydrate metabolism caused by thyroidectomy on the basis of mere blood sugar determinations, since much more detailed metabolic studies would be required before one could discuss this question to advantage. Yet it seems obvious that the rise in blood sugar is elicited by the non-specific damage inflicted upon the animal and not by the specific action of any of the agents employed; otherwise we should not have obtained the same result with such different agents as formaldehyde, cold, muscular exercise and even fasting.

It is hardly within the scope of this communication to review the existing rather voluminous and contradictory literature on blood sugar changes following thyroidectomy. It is evident however that since a short period of fasting decreases, while a longer period of fasting or exposure to any other damaging agent increases, the blood sugar of the thyroidectomized animal, all experiments designed to study the specific effect of a certain agent on the blood sugar of a thyroidectomized animal necessarily had to be complicated by this particular change in the response to the non-specific harmful stimulus.

SUMMARY

Thyroidectomized rats respond with hyperglycaemia to non-specific damaging agents which decrease the blood sugar of the normal rat.

While a 24 hr. fasting period causes more pronounced hypoglycaemia in thyroidectomized than in normal rats, 48 hr. of fasting actually increase the blood sugar of the thyroidectomized rat while in the normal it causes a further decline in blood sugar concentration.

Thyroidectomized rats exposed to non-specific damaging agents respond with a more pronounced decrease in blood chloride than normal animals. They show hypochloraemia even under the influence of excessive muscular exercise which raises the blood chloride of the normal rat.

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CCLXVIII. THE CONCENTRATION OF COENZYME-LIKE SUBSTANCE IN BLOOD FOLLOWING THE ADMINISTRATION OF NICOTINIC ACID TO NORMAL INDIVIDUALS AND PELLAGRINS

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THE recent demonstration that nicotinic acid, or its amide, is the vitamin whose deficiency is responsible for canine black tongue and pellagra is of great theoretical interest, for the pyridine ring plays a role of the first importance in cellular respiration. This was demonstrated by Warburg *et al.* [1935] who found their coenzyme (triphosphopyridinenucleotide designated Co II) to comprise one mol. of nicotinic acid amide, three of phosphoric acid, two of (probably) pentose and one of adenine. The nucleotide in the presence of a suitable protein is reduced by the substrate, then reoxidized indirectly by molecular oxygen; this cycle of oxidation occurs in the pyridine ring. Cozymase (diphosphopyridinenucleotide, Co I) differs from Co II only in possessing one mol. less of phosphoric acid, according to Warburg & Christian [1936] and Euler *et al.* [1936]. The mechanism of its cycle of oxidation is the same.

It is almost certain that all active tissues in the human body contain Co I and Co II, and consequently one would expect that variation in amount of dietary nicotinic acid should lead directly to variation in amount of coenzyme. It is the object of this paper to demonstrate that the concentration of coenzyme, or of some closely related substance in the blood, is sensitive to the ingestion of nicotinic acid, both in normal individuals and pellagrins.

The vitamin action of nicotinic acid was first reported for bacteria. Lwoff & Lwoff [1937] proved that Co I or Co II could replace the *V*-factor of the haemophilic bacteria. At about the same time nicotinic acid was shown to be a growth factor for *Staphylococcus* by Knight [1937] for the diphtheria bacillus by Mueller [1937], and recently for the dysentery bacillus by Koser *et al.* [1938].

Frost & Elvehjem [1937] reported that adenylic acid plus nicotinic acid (1 mg. per day of each) completed a basal diet on which rats did not otherwise grow, and Euler *et al.* [1938] obtained a striking response by the addition of 1 mg. per day of Co I (cozymase). However, rat dermatitis (and chick dermatitis) is not due to a nicotinic acid deficiency according to Dann [1937].

Elvehjem *et al.* [1937; 1938] showed that nicotinic acid or its amide is the vitamin whose deficiency is responsible for black tongue in dogs; this has been confirmed by Street & Cowgill [1937] and others, and extended to a pellagra-like condition in swine by Chick *et al.* [1938]. Pellagra, like black tongue, has yielded to nicotinic acid therapy according to Fouts *et al.* [1937], Smith *et al.* [1937], and Spies *et al.* [1938]. Schmidt & Sydenstricker [1938] reported relapses following rapid improvement with nicotinic acid therapy, but neither the diet nor the activity of the patients was controlled, so that at present their results are inconclusive [*vide* also review by Sebrell, 1938].

Method

The concentration of coenzyme-like substance, which is confined to the corpuscles, was determined with the aid of *Haemophilus parainfluenzae*, the growth of which under proper experimental conditions is proportional to the coenzyme content of the culture broth.

Specificity of test

Lwoff & Lwoff [1937] suggested the use of *H. parainfluenzae* for the bioassay of coenzyme when they found that:

(1) Either Co I or Co II at concentrations as low as about $2 \times 10^{-9} M$ can replace the *V*-factor required for growth by all *parainfluenzae*.

(2) *V*-factor cannot be replaced by adenylic acid (yeast or muscle), nicotinic acid, nicotinamide, diethylamide, *o*-dihydropropylnicotinamide, or the products formed when either coenzyme is kept at 100° and *p*H 8·5 for 20 min.

(3) The reaction Co I $\rightleftharpoons$ Co II can be accomplished by the bacteria when either coenzyme enters the protoplasm, after which its identity appears to remain fixed.

I have found that neither nicotinic acid nor its amide in therapeutically active concentrations, either alone or in the presence of all substances found in the blood extract used for assay, is able to act as *V*-factor, as the following examples show. (A) The addition to blood extract of 1 mg. acid or amide per ml. blood reduced the assay by 4 and 6% respectively. (B) Incubation of whole blood containing 1 mg. acid per ml. at 37·8° for 1 hr. increased the assay by 6%. (C) Incubation of whole blood containing 10 mg. acid per ml. rapidly produced methaemoglobin, haemolysis and loss of *V*-factor.

Products of the mild acid hydrolysis of *V*-factor were found to be inactive, as when blood, diluted 100 times, was boiled for 20 min. in 0·8 *N* trichloroacetic acid containing 0·55 *N* NaOH.

These results are in harmony with those of the Lwoffs; they argue strongly in favour of the unique identity of Co I and Co II with *V*-factor, and prove that only a special and limited group of compounds containing nicotinic acid, or its amide, can be active. However, the terms coenzyme and *V*-factor will not be used interchangeably in the following, and the substance or substances measured in the bio-assay will be referred to as *V*-factor.

Theory of test

When broth is inoculated, the optical density of the resulting suspension increases owing to an increase in living and dead bacterial matter, and to changes in the medium itself. The increase in light adsorption is rapid at first, but reaches a maximum at which it tends to remain 24–30 hr. after inoculation, declining slowly thereafter. To measure directly the change in light transmission of a culture it is most convenient to employ the Evelyn photoelectric colorimeter [Evelyn, 1936], which uses test tubes for absorption cells.

Let T_b be the transmission of the blank culture, and
  T_x the transmission of the experimental; then
  a the % change in absorption is given by (1):

$$a = 100 \left[1 - \frac{T_x}{T_b} \right]. \qquad \qquad \ldots\ldots(1)$$

V-content is found by determining the amount of blood necessary to produce a value of a equal to that produced by a standard solution.

Let a_s be some set value of a used as a point of reference,

X the concentration (ml./ml.) of blood in the unknown test whose reading is a_s,

S the concentration (ml./ml.) of standard V-factor in the standard test whose reading is a_s, and

H the haematocrit reading (volume of red and white cells expressed as $^o/_o$ blood volume); then

V the concentration (units/ml.) of V-factor in the blood cells, expressed in arbitrary units and multiplied by 10^4 for convenience, is given by (2):

$$V = \frac{10^4 \, S}{HX}. \qquad \qquad \dots\dots(2)$$

V determined according to (2) will be used as an empirical measure of the concentration of Co I and Co II, bearing in mind, however, that some unknown substance closely related to the coenzymes might give a positive test. No attempt was made to distinguish between the two coenzymes.

Procedure of test

The cultures are prepared in tubes suitable for use in the photoelectric colorimeter, each containing 7 ml. broth. To one series of tubes in duplicate there are added 0·0, 0·1, 0·1, 0·2 and 0·3 ml. blood extract; to a second series there are added similar amounts of standard V-factor solution. All tubes are inoculated except the second pair in each series. The tubes are checked in the photometer (using filter 520 M) for initial differences in absorption, which are noted and used as corrections. After 24 and 30 hr. of incubation at 37° they are read in the photometer; the maximum value of a is used, and the averages for each pair are plotted as in Fig. 1. Usually the difference between the 24 and 29 hr. readings is small and the duplicates agree fairly well as in the following readings made at 24 hr.:

Standard V. 0·1 ml., 2, 1·5; 0·2 ml., 7, 7; 0·3 ml., 11·7, 11.

Blood extract. 0·1 ml., 3·3, 4; 0·2 ml., 11·2, 12·2; 0·3 ml., 14, 13.

The horizontal bar cutting the two curves in Fig. 1 represents a_s, i.e. the value of a used in comparing the unknown and standard solutions. a_s equal to 9 has been used throughout the present work. In Fig. 1 we find by interpolation that 0·16 ml. blood extract is equivalent to 0·245 ml.

Fig. 1. a as a function of V-factor (in ml.). Open circles, V-factor from blood extract; solid circles, V-factor from yeast extract used as a standard. The bar cutting the two curves at $a = 9$ (designated a_s) facilitates comparison: 0·16 ml. blood extract is equivalent to 0·245 ml. yeast standard.

standard V-factor solution (for preparation and concentration, see below), each producing a change equal to a_s. From these data X and S are calculated, and knowing H the value of V is obtained from (2).

Blood extract. Blood obtained by venepuncture is prepared by the addition of 1·2 mg. potassium oxalate per ml. 0·1 ml. blood is added to 10 ml. distilled water. Immediately 2 ml. are withdrawn for the haemoglobin determination [Evelyn, 1936] and are replaced by 2 ml. trichloroacetic acid solution (13% acid containing 2·15% NaOH). The test tube is plugged, centrifuged after several minutes and stored on ice. The V-factor is contained in the supernatant liquid, which loses 5% of its activity in 24 hr. Plasma prepared in this manner supports no measurable growth, even when 1 ml. instead of 0·1 ml. is used.

Cultivation of bacteria

The bacteria used were of the same strain as those employed by the Lwoffs, namely, *H. para-influenzae* strain 4101 Fleming, National Collection of Type Cultures, Lister Institute, London. Subcultures were made at least three times a week, alternating between broth and agar. When first received, the bacteria showed 50–100 % more growth if glucose or sucrose were added to the medium. After 3½ months of cultivation at 37°, however, this phenomenon disappeared rather suddenly, although the ability to oxidize sugar was retained. All data reported here were obtained after the mutation had occurred; during this period cultivation in broth was almost continuous.

V-factor. This is prepared by suspending 100 g. baker's yeast in 50 ml. water, pouring the suspension into 150 ml. boiling water containing 6·8 g. KH_2PO_4, and maintaining the temperature at 80–85° for 20 min. The suspension is then filtered through a bacteriological paper, and sterilized by passing through a candle or a Seitz filter pad. The concentration of this solution is 5×10^{-1}, as 0·5 g. yeast per ml. water has been used. The strength of the *standard solution* referred to in the text and used in all experiments was 6×10^{-4}.

Medium. A 2 % solution of proteose-peptone (Difco) containing 0·6 % NaCl is titrated with NaOH to pH 7·7–7·8, tubed in 6 ml. samples, and autoclaved. Sterile *V*-factor is then added. The usual subculture receives 0·1 ml., concentration 5×10^{-2}. Subcultures intended to supply inocula for blood tests receive 0·1 ml., $2·5 \times 10^{-3}$, all of which will be used up after 24 hr. of growth. Thus when the test culture is inoculated with one drop, no *V*-factor will be carried over. For a solid medium, the above is used plus 3 % agar (*V* added when agar has cooled, but before solidification).

EXPERIMENTAL RESULTS

Figs. 2, 3 and 4 together with Table I summarize the experimental data. The solid black points are plotted for periods during which nicotinic acid was taken; an arrow indicates when the administration of acid began or ended between two points; *V* the ordinate in each graph was calculated according to equation (2), *H* and *S* being redetermined for every point. The abscissae of all are comparable; i.e. a determination made on the twentieth day of Fig. 2 would have been made simultaneously with that on the twentieth day of Fig. 3. This was done to provide a further check on the method.

All normal subjects took nicotinic acid by mouth after breakfast and dinner either in tablets (Squibbs) or in solution (S.M.A. Corp. nicotinic acid dissolved in water). A 0·5 % solution (partially neutralized) has a slightly sour taste and is not unpleasant. When 100 mg. are taken on an empty stomach (e.g. before breakfast) a peripheral flushing occurs, the extent of which varies in different individuals. Usually within 5 min. the face and neck tingle, then flush, followed by the forearms and probably the knuckles and knees. In some cases intravenous injection was used.

Normal subjects

(1) HK (Fig. 2), white male, age 28, height 179 cm., weight 66·5 kg. During first 10 days, average *V* was 19. From 10th to 24th day about 100 mg. nicotinic acid were taken daily, the change in *V* being as follows: 16th day, total nicotinic acid 475 mg., +20 %; 19th day, 825 mg., +40 %; 22nd day, 1500 mg., using smooth curve +50 %.

Beginning with the 24th day nicotinic acid was discontinued, and the subject was placed on a pellagra-producing diet (defined below). The increase in *V* declined: 32nd day, +10 %; 40th day, +0 %. Beginning on the 43rd day 1200 mg. nicotinic acid were taken within 24 hr. (300 mg. at 11.30 a.m., 2 p.m., 6.15 p.m. and 10.30 a.m.): 8 hr. after first dose *V* rose +10 %; 24 hr. (44th day), +40 %; 48 hr. (45th day), +50 %; 5 days (48th day), +30 %; 9 days (52nd day), +5 %.

The pellagra diet used was similar to that of Ruffin & Smith [1937], but with the following changes: *omitted*, cod liver oil, iron ammonium citrate, calcium gluconate, field (cow) peas, lard and flour; *decreased*, ascorbic acid to 50 mg. per day; *added* per day, 1 mg. aneurin-Cl; 5 drops oleum percomorphum, 200 g. potatoes, 5 stewed prunes, 70 g. apple sauce, 20 g. butter. The modified diet, as compared with the original, contains less preventive substance [cf. food ratings of Sebrell, 1934], but is low in protein.

(2) RM (Fig. 3, open circles), white male, age 24, weight 70 kg. Average initial V was 17 during the 13th–16th days. During 16th–23rd days about 100 mg. nicotinic acid taken daily. The changes in V together with the total dose were: 20th day, 400 mg., $+30\%$; 23rd day, 1 g., smooth curve $+35\%$; thereafter nicotinic acid stopped, 34th day, -5%. Beginning on the 50th day 1·25 g. nicotinic acid were taken during 24 hr. The changes in V were: 50th day (after 250 mg.), $+10\%$; 52nd day, $+30\%$; 55th day, $+25\%$.
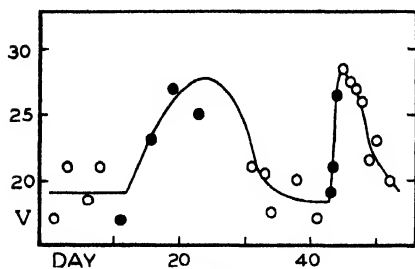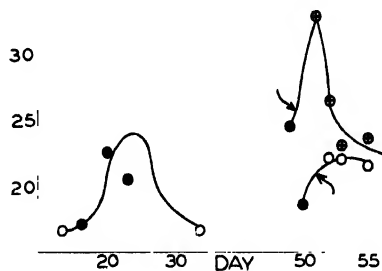
Fig. 2.   Fig. 3.

Fig. 2. Variation of V in the normal subject HK. 1·5 g. nicotinic acid taken during the 10th–24th days produced an increase of 50%. 1·2 g. taken within 24 hr. on the 43rd day produced a similar effect. Solid circles indicate periods during which nicotinic acid was taken.

Fig. 3. Variation of V in the normal subject RM (open circles) and the pellagrin JH (crossed circles). Solid circles indicate the administration of nicotinic acid, which was stopped at the arrows. RM (left curve) received 1 g. nicotinic acid in 7 days during which V rose 35%. Later (right-hand curve) RM received 1·25 g. in 24 hr., and V rose 30%. JH the pellagrin received 700 mg. nicotinic acid within 48 hr. ending at the arrow. Note the rapid rise in V of 35%, and the rapid fall.

The remaining four white male subjects, whose data are graphed together in Fig. 4C, will be considered as a group. Ages, weights and graph symbols are as follows: FE, 23, 66, circle; GS, 24, 61·5, triangle; KB, 22, 79·5, square; WK, 22, 97·5, inverted triangle. The daily dose of acid was about 1·5 mg. per kg.; FE and GS took 100 mg. per day, KB 125 and WK 150.

The data show an average initial V of 18 during the 13th–16th days. About 1·5 mg. nicotinic acid per kg. were taken daily during the 16th–20th days, and twice this dose for the 21st and 22nd days. The increases in V were: 20th day, 40%; 23rd day, 45%; 35th day, 10%.

Pellagrins

(1) JH, white male, age 55, weight 40 kg. The patient had suffered from oesophageal obstruction for 25 years. For 6 months before admission almost all food eaten had been regurgitated within 5–20 min.; as a result he was quite emaciated. About 2 weeks before admission definite signs of pellagra had appeared. On admission, physical examination showed the dorsum of each hand to be red and rough, and the tibial surfaces of the legs to be discoloured with a brownish pigmentation. The lips were dry, the tongue very red and smooth, the buccal surfaces red, the pharynx red. Oesophageal dilatation was performed with marked relief of the obstruction, and the patient was put on a high caloric, high vitamin diet. Valentine's liver extract, 30 ml., t.i.d., and nicotinic acid, 20 mg., t.i.d., were given for 4 days, then stopped. On the 5th and 6th days 300 and 400 mg. nicotinic acid were given. Eight days later acid and liver extracts were restored to the diet.

Data for JH are plotted in Fig. 3, crossed circles; the arrow marks the end of 2 days during which 700 mg. nicotinic acid were taken. At the time of the first test 600 mg. had been taken already, and V was 24·5 (49th day on graph). Note the subsequent sharp rise and fall: $+35\%$, 51st day; $+8\%$, 52nd day. The true initial value of V is not known; hence the increase (see Table 1) is a minimum. The patient's course was one of steady improvement. The signs of dermatitis disappeared almost entirely within 3 weeks.

The following cases were kindly brought to my attention by Drs D. T. Smith and J. M. Ruffin; they constitute part of a series studied in other respects by these workers, which will be described

elsewhere. The basal diet referred to [Ruffin & Smith, 1937] contained no vitamin fortification and a minimum of *pp* factor.

(2) RC, white male, age 41, weight 61·9 kg., height 176 cm. Three weeks before admission, after exposure to sun, dermatitis developed on his hands, and was followed 1 week later by stomatitis and glossitis. Diet was fair, but lacking in meat. There was no history of alcoholism. On admission, physical examination showed the skin of the dorsa of the hands to be reddened, cracked and peeling. "Sand paper skin" was noted on both sides of the nose around the sebaceous glands. The gums were sore and tender, the mucous membranes, pharynx and tongue were red. Gastric analysis revealed no free HCl at any time. Gastroscopy showed a reddened mucosa without ulcers or erosions.

RC was placed on the basal diet and given graduated doses of sunlight to the right hand. After 3 days his tongue became red and swollen, the dorsum of the hands inflamed, and a mild diarrhoea developed. During this period the average V (Fig. 4B, circles) was 15. Nicotinic acid administration then began (3rd day in Fig. 4B), 90 mg. per day i.v. After the second dose an improvement could be noted in the glossitis, and from then on the patient improved steadily, although the sebaceous glands over the nose were tardy in responding. According to Fig. 4B the increases in V correlated to total dose of nicotinic acid were: 6th day, 270 mg., 40%; 9th day, 540 mg., 75%; 12th day, 810 mg., 85%.

In Fig. 4A there are compared the results obtained with two subjects on the same hospital diet and with same initial value of V, one of whom received nicotinic acid.
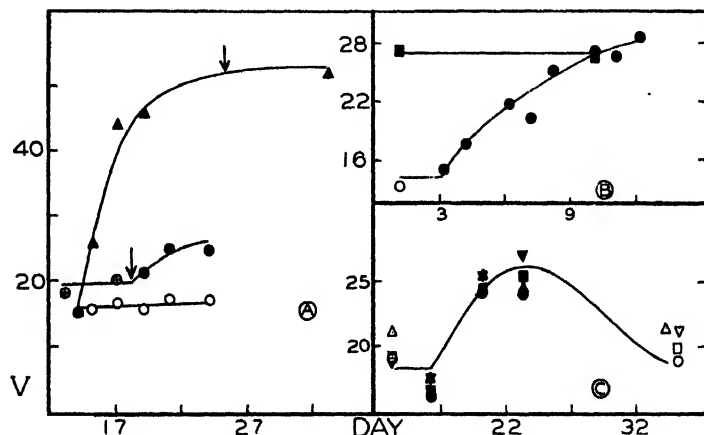
Fig. 4. Variation in V following the administration of nicotinic acid (indicated by solid circles and arrows). Fig. 4A, triangles, pellagrin JS, 1 g. daily, V rose 250%; crossed circles, pellagrin LL, 60 mg. daily, V rose 30%; open circles, pellagrin RJ, spontaneous recovery, V rose 5%. Fig. 4B, circles, pellagrin RC, 90 mg. daily, V rose 100%; squares, pellagrin JA, received 5·6 g. during 26 days before first determination, and a total of 8·8 g. at time of the second. Fig. 4C, normal subjects, circle, FE; triangle, GS; square, KB; inverted triangle, WK. Each took 1–1·5 g. nicotinic acid. The average rise in V was 45%.

(3) RJ, white male, age 55, weight 61·3 kg., height 169 cm. The diagnosis was pellagra, hypertensive cardiovascular disease and arteriosclerosis. Inquiry revealed that he had had an apoplectic seizure 17 months previously, following which his physician had ordered the elimination of meat from the diet. There was also a lack of fruit. Two weeks previously after exposure to the sun for several hours the signs of pellagra had appeared. When admitted, the dorsal skin of each hand was fiery red and desquamating, with oedema of the subcutaneous tissue. The bowels were costive. Glossitis was not present.

The data for RJ are plotted in Fig. 4A, open circles. When admitted, he was placed on the basal diet and exposed to the sun for several days without effect. The dermatitis improved steadily, and he was finally discharged some 20 days later. The initial value of V was 15, and it remained at this level, advancing to 16, an increase of about 5%. The spontaneous recovery may have resulted in part from the eating of a meal of liver following the outbreak of the dermatitis.

(4) JS, white male, age 55, weight 59·9 kg., height 158 cm. The diagnosis was pellagra, hypertensive vascular disease, generalized arteriosclerosis and peripheral neuritis. About 2 years previous to admission, the patient had been treated at this hospital for pellagra with Valentine's liver extract, with excellent results. Upon returning home, however, medication was stopped and meat was eliminated from the diet because of the high blood pressure. One month before admission, he had strolled through the fields in the sun; shortly afterwards dermatitis, stomatitis and diarrhoea developed. Upon admission, dermatitis of the dorsum of the hands was noted, the lips were swollen and sore, the mucous membranes red and dry, the tongue bright red and very slick and the pharynx reddened. There was no diarrhoea, but the patient complained of gastrointestinal symptoms. which continued throughout his course in the hospital. Gastric analysis showed the presence of free HCl after histamine. The patient was put on the basal diet, and 2 days later the oral administration of nicotinic acid was begun, 250 mg., q.i.d. Beginning 3 days after admission, and continuing for 3 successive days, 100 mg. per day of aneurin-Cl were given.

Data for JS are plotted in Fig. 4A, triangles. The initial value of V was 15; graph abscissa, 14th day. Thereafter the total dose of nicotinic acid and increases in V were: 15th day, 1 g., 75%; 18th day, 4 g., 190%. Administration of nicotinic acid was stopped on the 25th day, total dose, 11 g. On the 33rd day, the increase in V was 245%; probably the increase was even greater around the 27th day.

(5) JA, white male, age 65, weight 56·6 kg., height 177·5 cm. Diagnosis: pellagra, generalized arteriosclerosis, polyneuritis due to beriberi. At time of admission this patient was critically ill. He was afflicted with a marked psychosis. the lesions on his hands were secondarily infected and the stomatis and glossitis were marked. He was put on the basal diet and given nicotinic acid, 90 mg. i.v. daily for 10 days. At the end of this period there was an amazing improvement in his entire condition. Nicotinic acid was then given orally, 90 mg., q.i.d., and also aneurin-Cl.

The first value of V for JA (Fig. 4B, squares) was obtained 26 days after treatment had been begun, at which time the patient had received 5·6 g. nicotinic acid and 450 mg. aneurin-Cl; V was

Table I. *Effect of nicotinic acid administration on* V

Subject	Total acid taken	Period of administration	Initial	Maximum	Increase	Duration of increase
Normals	mg./kg.	days	units	units	%	days
HK*	22·5	10	19	28	50	10
	18	1	19	28·5‡	50	8
JM	14·5	7	17	23·5	35	11
	18	1	(17)	22‡	30	—
Average of FE, KB, GS WK	14·5	7	18	26·5	45	8
Pellagrins						
JH	17·5	2	Less than 24·5	33§	More than 35	2
RC†	4·3	3	14	21	50	—
	8·6	6	—	26	85	—
	13	9	—	28	100	—
JS†	17	1	15	26	75	—
	67·5	4	—	44	190	—
	186	11	—	52‖	254	—
LL	8	6	19	25	30	—
JA	215	26	—	26·5	--	—
	250	35	—	26·5	---	—

* After taking first dose of nicotinic acid the subject was maintained on a pellagra-producing diet.
† On Ruffin & Smith basal diet.
‡ 24 hr. after nicotinic acid was discontinued.
§ 36 hr. after nicotinic acid was discontinued.
‖ 8 days after nicotinic acid was discontinued

about 26·5; 9 days later the value was the same, the total doses being 8·8 g. nicotinic acid and 850 mg. aneurin-Cl.

(6) LL, white female, age 40, weight 45·8 kg., height 160 cm. Diagnosis: malnutrition, pellagra, secondary anaemia, pyorrhea alveolaris, chronic cervicitis. Three months before admission there had been a gradual onset of weakness, anorexia and proneness to fatigue; 3 weeks before admission dermatitis began. When admitted, the skin of the face was somewhat reddened, and on the hands and arms to just over the elbows it was rough, brownish and scaling. Similar lesions were present on the ankles.

LL was put on the basal diet and exposed to sunlight, but showed no sensitivity. The data for V are plotted in Fig. 4A, crossed circles. The initial value was about 19; after 6 days on a high caloric, high vitamin diet plus 60 mg. nicotinic acid per day (begun at arrow), V had risen 30%. The patient's general condition improved and she was discharged.

DISCUSSION

The results presented, which are plotted in the figures and summarized in Table 1, establish that V is a direct function of nicotinic acid intake. The normal value is about 18 ± 2, and this was increased by as much as 200% following the ingestion of nicotinic acid. Within the range of dose studied (about 20 mg. per kg.), the increase in V is independent of the duration of the period of administration (1–10 days).

It is rather surprising, however, that V should fall so rapidly to the original basal level when nicotinic acid is discontinued, particularly since no measurable quantities are detected in the plasma. Apparently some fundamental difference exists between the initial V-factor found in the corpuscle and the increment which is rapidly added. The basal level is stable and possibly is determined entirely at the time of haematopoiesis, whereas the increment is mobile and reflects the current state of nutrition.

Whether these levels can be used to indicate pellagrous conditions is not certain. It is suggestive that the duration of the increment in the pellagrin JH was 2 days, whereas in the normals it was about 10. The relation between the basal level and pellagra is certainly complicated. For example, RJ and JS (Fig. 4A) had the same initial V although the symptoms of JS were very much the worse; RJ made a spontaneous recovery. The few cases reported taken with several others indicate that pellagra is to be suspected when V is not more than 15, although the converse may not be true.

No relation between V and haemoglobin was noted.

If we accept nicotinic acid as the vitamin whose deficiency causes pellagra, a number of consequences follow.

(1) In pellagra the coenzyme level of the body will be reduced, leading directly to a reduced capacity for oxidations and reductions. The basal metabolism will not necessarily be affected, however, since a fraction of the total coenzyme should be adequate for it, but the maximum rate of work will be decreased.

(2) The more active the organism, the greater will be its nicotinic acid requirement. This conclusion is attractive because it will explain some of the spontaneous recoveries, and also because it partly accounts for the differential susceptibility of individuals on the same diet.

The coenzyme role of nicotinic acid, however, does not appear capable of explaining all of the facts relating to pellagra. As noted previously, the relationship between blood V-level and pellagra is not clear. Furthermore, since these experiments were completed, Daft et al. [1938] in a preliminary note have

reported that 50 mg. of Co I administered intravenously failed to have a therapeutic effect on black tongue. Thus it seems probable that nicotinic acid enters the metabolism in ways as yet unknown.

SUMMARY

The bio-assay of blood V-factor was accomplished by means of *Haemophilus parainfluenzae*, as supplied by Lwoff & Lwoff. V-factor represents the coenzyme moiety (di- and tri-phosphopyridine nucleotides), and, possibly, closely related substances which are unknown. Nicotinic acid (or amide) does not give the test.

V-factor is confined to the corpuscles. About 20 mg. nicotinic acid per kg. taken in 1–10 days will increase V by 35–75% in 2–10 days in normal individuals and pellagrins.

The rapid rise in V-factor is followed by a fairly rapid fall to the original level. Apparently, material responsible for the increment in V is to be distinguished from that which constitutes the initial corpuscular content.

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CCLXIX. MECHANISM OF SYMBIOTIC NITROGEN FIXATION

IV. SPECIFIC INHIBITION BY HYDROGEN[1]

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BECAUSE of the practical importance of the problem in agriculture, research on the fixation of atmospheric nitrogen through association of the root nodule bacteria and leguminous plants has been primarily directed towards answering questions of immediate agronomic significance. As a result, advances concerned with the more fundamental aspects of this biological process have lagged behind practical applications. Part of the lag, however, may be ascribed to lack of suitable techniques for investigation of the biochemical phases of the problem. In symbiotic nitrogen fixation the life processes of two organisms, plant and bacteria, are intimately related so that there is great difficulty in isolating the factors which have special significance for the fixation reaction. This difficulty has been overcome in part by Wilson and collaborators [Wilson, 1936; Wilson & Fred, 1937; Wilson & Umbreit, 1937] through the application of a physico-chemical approach to the study of the properties of the enzyme system responsible for the fixation.

In the initial studies, which were undertaken to determine the influence of the pN_2 in the atmosphere on the fixation reaction, it was observed that the partial pressure of N_2 could be lowered to about $0.10-0.15$ atm. without decrease in the quantity of N_2 fixed by nodulated red clover plants provided that the N_2 removed was either unreplaced or replaced with helium or argon. If the N_2 were replaced with H_2, however, the total quantity of N_2 fixed decreased linearly with the pN_2 of the atmosphere throughout the range 0.06 to 0.80 atm. As such a finding suggests that H_2 may be a specific inhibitor for the symbiotic N_2 fixation process, detailed studies of the "hydrogen effect" were undertaken.

Of primary importance is the demonstration that the effect of H_2 on the assimilation of free N_2 is quantitatively different from the effect on the uptake of combined forms, as NH_4^+ and NO_3^-. A previous report [Wilson & Umbreit, 1937] discussed the various types of experiment which might be expected to throw light on this question; in the same report data were summarized from experiments in which the *total quantity* of N_2 fixed was used as a measure of the effect of H_2. In this paper other data will be presented from experiments in which the *rate* of fixation is used as a criterion.

Methods

Twenty red clover plants (*Trifolium pratense*, L.) were grown in a N-poor sand substrate placed in 9-l. pyrex pressure bottles. All plant nutrients except combined N were added to the sand. Plants of one series were inoculated with an effective strain of the specific organism, *Rhizobium trifolii*; plants of a second series were not inoculated but furnished periodically with combined forms of N.

Under the conditions of the experiments the limiting factor for development of the plants was the supply of N. Each bottle was closed with a gas-tight rubber stopper fitted with connexions for changing the atmosphere given the plants. Details of the methods have been described previously [Wilson, 1936].

General considerations of the experiments

As was emphasized in an earlier paper [Wilson & Umbreit, 1937], rate experiments with plants grown in a controlled environment present technical difficulties associated with restriction of available space and apparatus. In most experiments there were six treatments (three different partial pressures of H_2 with two series of plants); at each harvest cultures from each treatment were harvested in duplicate. Since in this work not more than about 50 plant cultures could be made in any one experiment, the maximum number of harvests which could be taken was four. The period during which the plants were under different atmospheres rarely exceeded a month; at the end of this time the inoculated plants in air (or plants which had been given combined N) completely filled the plant container. As it was inadvisable to make a harvest more frequently than once a week, this consideration once again imposed a limitation of four harvests.

When the quantity of free or combined N assimilated by red clover during the period under study is plotted against time, the resulting curves are logarithmic in form; when plotted on semi-log paper the points fall along a straight line. The following factors will cause minor deviations from this straight line.

(1) *Sampling errors.* In spite of all efforts to maintain the plants in identical environments other than the composition of the atmosphere furnished them, variations between the N content of replicates reached 10–15%. Statistical analysis of a large number of experiments [Wilson, 1936; Wilson & Umbreit, 1937] indicates that the *mean* of duplicate samples has a standard error of 5 to 8%. In rate experiments if only duplicate samples are taken at each harvest, through chance these may be cultures with N content definitely lower (or higher) than the mean of all the samples of that particular treatment. An inaccurate sample at one harvest will affect succeeding samples in an opposite direction since the remaining samples will be higher (or lower) than the general mean. For this reason it is believed that the best straight line which can be drawn through the experimental points probably represents the true course of development of the plants. In calculations, points on this line are taken rather than the actual values derived from the samples.

(2) *Restriction of growth.* As has been mentioned, at the end of the experiment the plants in certain of the treatments almost fill the container. During the last week of growth it is probable that the development of these plants is restricted to some extent because of competition for space. This restriction will cause the observed value for N uptake to be less than it would have been had the competition not occurred. A partial remedy for this may be to grow fewer plants in the container, but a reduction in the number of plants taken for analysis will increase the sampling error so that little is gained. Moreover, a great deal of the restricted growth obtains because the plants have reached the top of the container and are more easily shaded by the stopper and other attachments on the bottle. Decrease in the number of plants per bottle would not correct this cause of restricted growth.

(3) *Change in environment.* In spite of the fact that the experiments are relatively short-time in nature, during certain seasons there may be encountered great variations in the weather which will affect the rate of development of the plants for short time intervals. Little can be done to avoid the vagaries in the climate other than to perform the experiments, in so far as is convenient, during seasons of fairly stable weather conditions.

If the development of the plants is logarithmic with respect to time, it is advantageous to use the specific constant of N assimilation, g, for comparison of effect of treatment [Burk, 1934]. This constant is defined by

$$g = 2 \cdot 303 \frac{d \log (a+y)}{dt} = 2 \cdot 303 \times \text{slope straight line of semi-log graph}$$

in which a equals N content of plants at start of treatment and y equals increase after time t. The value of this constant represents rate of assimilation of N per unit concentration of N and is therefore independent of the time.

Rate experiments

Two experiments have been made in which four harvests were taken, and three others in which there were three harvests. In the latter experiments another aspect of the problem was under simultaneous investigation (see next section) so that not all the plant cultures were available for the simple rate tests.

Exp. 1. This was made during the early autumn months, and the natural illumination was supplemented by artificial light. NH_4NO_3 was used as the source of combined N. The assimilation of N is shown in Fig. 1. From these data it is evident that the inhibition of N fixation by H_2 does not arise from mere restriction of fixation during the early stages of treatment but is an effect which persists throughout the experiment. In contrast to this effect of H_2 on the assimilation of elemental N the uptake of combined N is independent of the pH_2 in the atmosphere.
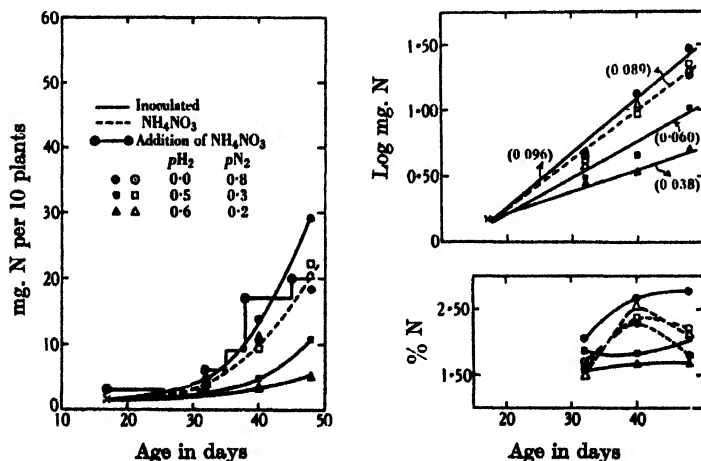
Fig. 1. Comparison of assimilation of free and combined N (NH_4NO_3) by red clover in atmospheres of various pH_2. Figures in parentheses represent g values.

(Exp. 1, planted 12 September; all cultures in air until harvest I, 29 September, then changed to atmosphere indicated; harvest II, 14 October; harvest III, 22 October; harvest IV, 30 October, 1936.)

When log mg. N is plotted against time, the fit of the points to a straight line is quite satisfactory except possibly of those for the atmosphere containing a pH_2 of 0·4 atm. Even in this case the deviations from the line hardly exceed sampling errors. The points corresponding to the plants given combined N fall along a single line, so the g values are identical for these plants whether H_2 is present or not. In contrast, the g values for plants dependent on the fixation of free N decrease with an increase in the pH_2 of the atmosphere.

The effect of H_2 on the % N in the plants should not be overlooked. With *inoculated plants* there is a marked decrease in the % N with increase in the pH_2 of the atmosphere. This means that N fixation is so restricted in these plants when grown in the presence of H_2 that carbohydrates accumulate. The appearance of the plants reflects this difference in composition; plants grown in air are

dark green in colour whereas those grown in an atmosphere containing H_2 have yellow leaves and red stems. With plants given combined N there is no association of % N with the pH_2, and the plants are indistinguishable in general appearance.

It is emphasized that the effect of H_2 on the N fixation process does not arise from the simultaneous decrease in the pN_2 of the atmosphere since in the range used the rate of fixation is independent of pN_2 [Wilson, 1936].

Exp. 2. As this was done during the summer months, light and other weather conditions were ideal for N fixation. The roof of the greenhouse was equipped with a water cooling system, and the bottles were kept in a trough of flowing water in order to avoid excessive temperatures. Under these conditions the development of the plants was most satisfactory. In Exp. 1, through control of the addition of NH_4NO_3, the rate of assimilation of combined N was kept nearly equal to the rate of assimilation of free N_2 by the inoculated plants. The objection could be raised that such a procedure might mask an inhibition by H_2 of the uptake of combined N. If some other factor, such as supply of combined N, is restricting the growth of the plants, inhibition by H_2 might not be obvious. In Exp. 2 $(NH_4)_2HPO_4$-N was always supplied in excess so that the rate of uptake was not restricted because of low concentration of available combined N. $(NH_4)_2HPO_4$ was used, as other experiments had indicated that development of clover was less erratic with this form of N [Wilson & Umbreit, 1937].
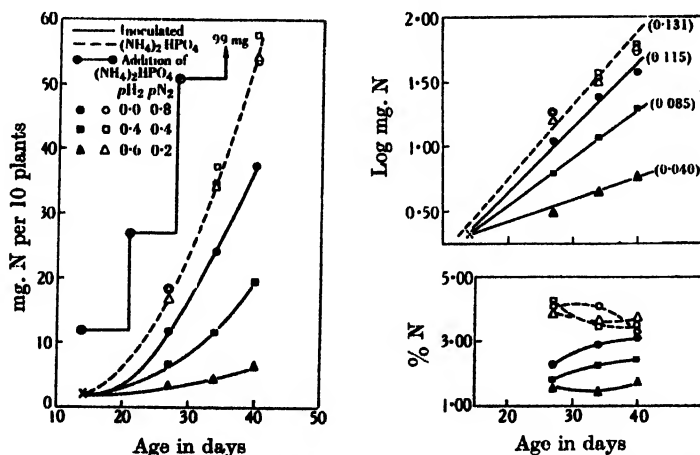
Fig. 2. Effect of pH_2 in atmosphere on assimilation of free and combined N ($(NH_4)_2HPO_4$) by red clover.

(Exp. 2, planted 6 July 1937; all in air until harvest I, 20 July, then changed to indicated atmospheres; harvest II, 2 August; harvest III, 9 August; harvest IV, 15 August.)

The data given in Fig. 2 confirm in every way the conclusions discussed in connexion with Exp. 1. The fit of the experimental points to the straight lines in the semi-log plotting is very satisfactory. These results point to an unmistakable inhibition of the fixation of free N by H_2, an inhibition which is not evident in the assimilation of combined N.

Exps. 3 and 4. These are a part of the series to be discussed in the next section in which the treatment was changed during the course of the tests. Only the semi-logarithmic plots (Fig. 3) are given since these summarize the results of chief interest. Data concerned with the % N were similar to those given in

Figs. 1 and 2. It will be noted that in these experiments the plants receiving combined N were also inoculated. Unless combined N is maintained at a high level, the plants will obtain part of their supply of this element through fixation. In these particular experiments it was desired to keep the rate of development of the plants in the combined N series close to that of the plants dependent entirely on fixation for their N. This was accomplished, as is shown by the g values given in Fig. 3, but the rate of adding NH_4NO_3-N did not entirely prevent fixation of
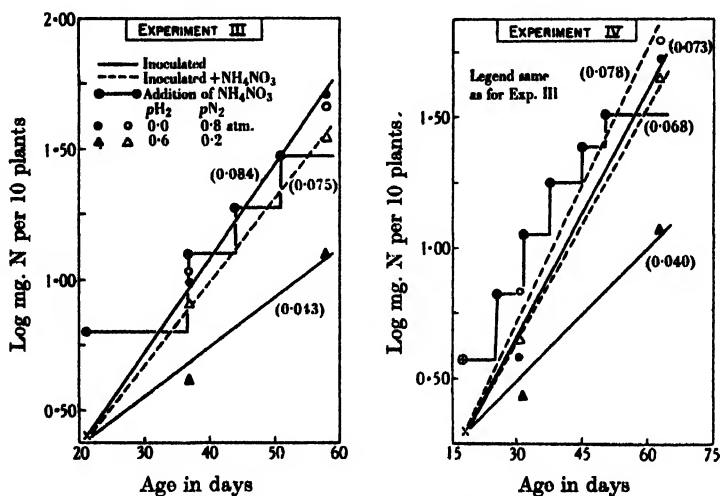
Fig. 3. Effect of H_2 on assimilation of free N_2 and NH_4NO_3 by inoculated red clover plants.
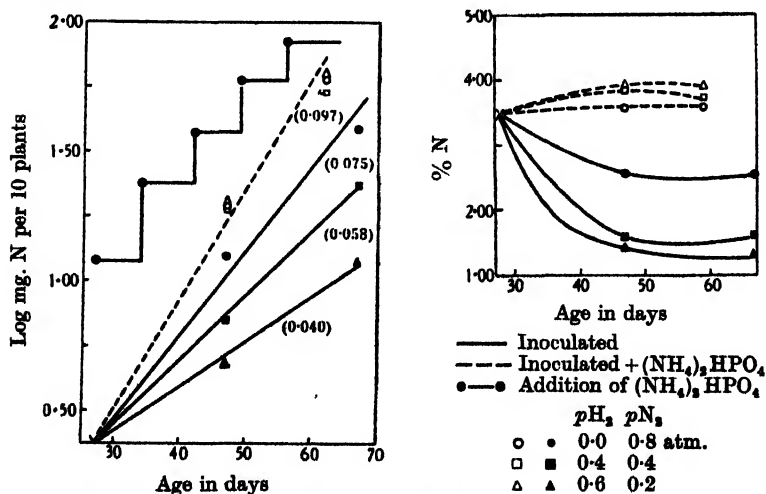
Fig. 4. Effect of presence of H_2 in atmosphere on assimilation of free N_2 and $(NH_4)_2HPO_4$ by red clover.

(Exp. 7, planted 19 August 1937; all kept in air until harvest I, 15 September; then changed to different treatments.)

elemental N by the plants of the combined N series. At harvest, well-developed nodules were found on all the plants. Under such circumstances, it would be expected that the plants of the inoculated plus combined N series which were

grown in the atmosphere containing H_2 would show some evidence of inhibition. As is shown in Fig. 3, such evidence was obtained; the g values for the plants supplied with c mbined N and kept in an atmosphere with a pH_2 of 0·6 atm. were slightly but significantly lower than those of similar plants kept in air.

Exp. 7. In this experiment the combined N, $(NH_4)_2HPO_4$, was added at such a rate that it was always in excess. As a result fixation of atmospheric N_2 by the plants of the inoculated plus combined N series was entirely suppressed, and the slight inhibition in the development of the plants because of presence of H_2 observed in Exps. 3 and 4 was not obtained (Fig. 4). Plants dependent entirely on fixation for their N showed the "hydrogen effect" both in rate of fixation and in % N in the plants.

The results of these five experiments concerned with the effect of H_2 on the symbiotic N fixation process in red clover consistently support the conclusion that inhibition of N fixation by H_2 persists throughout the growth of the plant and is not merely an initial effect associated with placing the plants in an atmosphere to which they are unaccustomed. The extents of inhibition by a pH_2 of 0·6 atm. as measured by the values of g were as follows: 57, 65, 49, 45 and 47 %. If the total quantity of N fixed is used as the criterion, inhibition reaches 65 to 75 % [Wilson & Umbreit, 1937]. The maximum inhibition was observed when the rate of development of the plant was greatest. If environmental conditions are not optimum for growth, fixation is restricted by other factors, e.g. a low supply of carbohydrate, and the extent of inhibition by H_2 is partially masked. Irrespective of the rate of growth of plants in air, the observed g values for plants kept in an atmosphere containing a pH_2 of 0·6 atm. were about 0·040. This means that H_2 was restricting fixation to such an extent that other factors which decrease fixation were relatively ineffective.

The most effective method for decreasing the growth rate of plants kept in air is to reduce the supply of carbohydrate, e.g. by shading. Such treatment would scarcely affect N fixation by plants grown in presence of H_2 since inhibition of fixation by this gas favours the accumulation of carbohydrate, an effect which would compensate for the reduced photosynthesis. The lowest value of g for plants grown in air for which clear-cut inhibition could be readily demonstrated would therefore be in the neighbourhood of 0·050–0·060.

In this connexion it appears that the range of growth rates which may be covered in this type of work is limited. In spite of growing the cultures under widely differing environmental conditions, especially with respect to intensity and duration of light, the g values for inoculated plants kept in the air varied only from 0·073 to 0·115. The g value of 0·115 was observed in an experiment in which the environment was close to ideal for maximum fixation of N. In this same experiment plants supplied with an excess of $(NH_4)_2HPO_4$ assimilated combined N at a rate equivalent to a g value of 0·131. It appears doubtful, then, whether a g value considerably higher than 0·115 could be obtained for fixation of N under the experimental conditions employed.

Change of treatment experiments

The purpose of these experiments was to show that inhibition of N fixation by H_2 was readily reversible and was not associated with any definite stage or rate of growth. In order to demonstrate reversibility of the inhibition, inoculated red clover plants were grown in an atmosphere containing H_2 until inhibition was evident. They were then either changed to an atmosphere free of H_2 or were kept in the H_2-containing atmosphere but supplied with combined N. That the

inhibition was not associated with any particular stage or rate of growth was shown by growing inoculated clover plants in air, then transferring to an atmosphere containing H_2 after fixation had proceeded for various periods of time. Occasionally, in these experiments one of the duplicate cultures of the combined N series would have to be discarded because of chance infection with algae.

Exps. 3 and 4. The data from these experiments are summarized in Table 1; briefly, they show that:

Table I. *Effect of change in atmosphere supplied to inoculated red clover plants on assimilation of free N_2 and NH_4NO_3 (Exps. 3 and 4)*

		Harvest I to II. Data for harvest II					Harvest II to III. Data for harvest III		
Atmosphere	NH_4NO_3	Total N* mg.	% N	g†	Atmosphere	NH_4NO_3	Total N* mg.	% N	g
Exp. 3:									
Air	–	9.8	2.80	0.084	Hydrogen‡	–	17.9	1.52	0.031
							19.6	1.48	0.035
Air	+	10.9	2.67	0.084	Air	–	47.1	2.95	0.077
							45.6	2.54	0.076
					Hydrogen	.	16.2	1.45	0.028
							22.9	1.52	0.044
Hydrogen	–	4.1	1.60	0.043	Air	–	29.8	2.70	0.087
							26.2	2.67	0.082
					Hydrogen	ǀ	30.0	2.50	0.088
							—	—	—
Hydrogen	+	8.3	1.91	0.075	Air	–	30.7	2.59	0.065
							52.8	3.00	0.090
					Hydrogen	--	18.8	1.55	0.041
							20.5	2.05	0.045
Exp. 4:									
Air	–	4.1	2.58	0.073	Hydrogen	–	19.3	2.07	0.043
		3.6	2.10				19.3	1.69	0.043
Air	+	7.2	3.00	0.078	Air	–	51.8	2.70	0.070
		6.5	2.46				58.8	2.68	0.074
					Hydrogen	--	25.8	1.85	0.047
							25.4	1.80	0.046
Hydrogen	–	2.72	2.08	0.040	Air	·	41.6	3.03	0.081
		2.18	1.58				32.2	2.42	0.073
					Hydrogen	+	22.3	2.63	0.061
Hydrogen	+	3.8	2.31	0.068	Air	·	60.5	2.81	0.081
		5.1	2.17				66.5	2.87	0.083
					Hydrogen	--	15.7	1.97	0.039

	Exp. 3	*Exp. 4*
Planted	3 March 1936	2 November 1936
Harvest I	8 April	20 November
II	24 April	3 December
III	15 May	4 January 1937

* Per 10 plants.

† These *g* values are taken from the lines of Fig. 3; they represent those for the cultures in which the treatments indicated in columns 1 and 2 were unchanged throughout the experiment. Similarly, the *g* values for harvest III are calculated from the points on these lines at harvest II, rather than the actual experimental points.

‡ Hydrogen = pH_2, 0.6 atm.; pN_2, 0.2 atm.; pO_2, 0.2 atm.

All cultures were inoculated and kept in air until harvest I, then changed to treatments indicated in columns 1 and 2, until harvest II, and finally changed to treatments indicated in columns 6 and 7.

(1) If inoculated clover plants are grown in air and then transferred to an atmosphere containing 0·6 atm. H_2, the rate of N fixation decreases from the high value characteristic of plants kept in air to that of plants which have been continuously maintained in an atmosphere of this pH_2.

(2) If clover plants which have been both inoculated and supplied with combined N are grown in air and after a period have their fixed N supply withdrawn, assimilation of free N proceeds at a rate characteristic of plants fixing N in air during the entire experiment. The slight decrease in the observed value of g probably arises from a lag in the development of the nodules occasioned by the presence of combined N in the early stages of development. If the plants are transferred to an atmosphere containing a pH_2 of 0·6 atm. when the supply of fixed N is withdrawn, fixation of elemental N is inhibited so that the g value with such plants drops to one equal to, or perhaps slightly higher than, that characteristic of inoculated plants given no combined N and kept in the H_2-containing atmosphere throughout the experiment. The slightly higher values occasionally encountered are most likely to be ascribed to residual combined N remaining in the substrate when the transfer is made.

(3) If inoculated plants which have been grown in an atmosphere containing H_2 on a substrate free of combined N are transferred to air, the rate of fixation increases from a low value to one that is typical of plants continuously kept in air. Moreover, if the plants are kept in the H_2-containing atmosphere but supplied with combined N, there is an immediate increase in the rate of assimilation to a value characteristic of plants supplied with combined N throughout the experiment.

(4) If inoculated clover plants are supplied with combined N and grown in an atmosphere with a pH_2 of 0·6 atm., the rate of N assimilation is only slightly less than that of similar plants grown in air (see discussion of Exps. 3 and 4 in the preceding section). If the supply of combined N is withdrawn, and the plants are kept in the same atmosphere, the rate of assimilation of free N assumes a value characteristic of inoculated plants grown in the presence of this pH_2. If, however, at the time of withdrawal of the combined N the plants are transferred to air, assimilation of free N proceeds at a rate typical of inoculated plants grown in air.

In all these various responses corresponding changes in the % N in the plants occurred coincidently with the changes in the rate of assimilation of N. It is clear from these results that the inhibition of N fixation by molecular H_2 is independent of the previous growth history of the plant and that the effect is readily reversible, that is, transfer from a H_2-containing atmosphere to air results in an increase in the rate of fixation and *vice versa*.

Exp. 5. In this experiment all plants were inoculated and kept in air until N fixation had begun, when all were placed in an atmosphere with a pH_2 of 0·6 atm. When inhibition of fixation had proceeded to the stage where the plants turn yellow because of excessive carbohydrate, four series of different treatments were formed. Plants of *Series 1* were transferred to air; those of *Series 2* were kept in the atmosphere containing H_2, but an excess of combined N as $(NH_4)_2HPO_4$ was added; plants of *Series 3* were treated as those of *Series 2* except that the combined N was supplied as $Ca(NO_3)_2$; plants of *Series 4* were kept in the H_2 atmosphere with no treatment. The essential data from this experiment which are shown in Fig. 5 provide additional evidence that the action of H_2 in the atmosphere is definitely concerned with the N fixation process and not with the general growth of the plant. If plants are returned to air after inhibition of fixation by H_2, the rate of N fixation soon reaches a value characteristic of fixation in air. This demonstrates that the effect of the H_2 is reversible. It is unnecessary, however, to return the plants to air in order to bring about normal development as this also can be accomplished by supplying combined N to the plants which are maintained in the atmosphere containing H_2. Coincidently with the increase in rate of fixation (or assimilation of combined N) after change in treatment there occurs an increase in the percentage N in the plants.
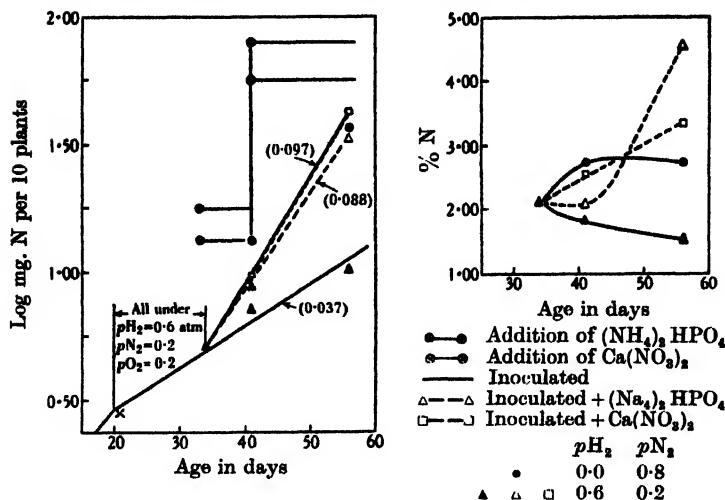
Fig. 5. Effect of change in treatment on assimilation of free and combined N by red clover.

(Exp. 5, planted 2 February 1937; all kept in air until harvest I, 19 March, then placed in H_2-containing atmosphere; treatments changed as indicated at harvest II, 2 April; harvest III, 19 April; harvest IV, 24 April.)

Table II. *Effect of source of N on inoculated red clover plants in presence and absence of H_2 (Exp. 6)*

Atmosphere	Source of N	Total N mg.	% N	g	Addition of combined N
Exp. 6:					
Air	N_2	30·9	3·12	0·152	2 Aug. —24·2 mg.
		30·9	2·96	0·152	NH_4^+–N
	$Ca(NO_3)_2$	16·5	3·62	0·108*	23·9 mg.
		27·6	3·61	0·144	NO_3^-–N
	$(NH_4)_2HPO_4$	42·8	3·44	0·176	10 Aug.—24·2 mg.
		32·9	3·81	0·157	NH_4^+–N
$pH_2 = 0·6$ atm.	N_2	5·34	1·73	0·028	23·9 mg.
$pN_2 = 0·2$ atm.		7·18	1·84	0·049	NO_3^-–N
$pO_2 = 0·2$ atm.		4·91	1·60	0·022	
	$Ca(NO_3)_2$	8·06	2·18	0·057*	
		33·8	3·51	0·157	
	$(NH_4)_2HPO_4$	26·0	3·45	0·140	
		28·5	3·57	0·147	

* See text for discussion of these values.

All cultures were inoculated and kept in air until harvest I; all changed to an atmosphere of $pH_2 = 0·6$, $pN_2 = 0·2$ and $pO_2 = 0·2$ atm. until harvest II; then to treatments indicated in columns 1 and 2 of the table.

Planted	6 July 1937
Harvest I	20 July (2·12)
II	2 August (3·63)
III	16 August

Figures in parentheses refer to nitrogen content of 10 plants at indicated harvest.

This experiment was made simultaneously with Exp. 2 (Fig. 2). The *g* values for cultures kept in air throughout experiment were:

Inoculated, 0·115;
Inoculated plus $(NH_4)_2HPO_4$, 0·131.

Those for cultures kept in pH_2 0·6 atm. were:

Inoculated, 0·040;
Inoculated plus $(NH_4)_2HPO_4$, 0·131.

Exp. 6. This is of chief interest as it illustrates a type of variation in response to change in treatment which is occasionally encountered, viz. delay in the recovery of plants on addition of combined N after these have been kept under a rather high pH_2 for some time. The experiment, the general plan of which was identical with that of Exp. 5, was conducted during the summer under conditions which favoured a high rate of photosynthesis. As a result the plants which had been kept in the H_2-containing atmosphere developed an excessive carbohydrate-nitrogen balance as evidenced by yellow leaves and red stems. Inoculated plants transferred to air responded immediately to the change in treatment, and in a few days the colour of the plants was changing to green. N fixation was evidently stimulated by the excessive carbohydrate [Wilson, 1935] since the value for *g*, 0·152, was the highest obtained in these experiments for assimilation of free N_2 (see Table II). The response of the plants given an excess of combined N (which would effectually prevent fixation of the element) was somewhat erratic. Uptake of the combined forms started immediately in the majority of the plants, but two cultures which were furnished $Ca(NO_3)_2$ did not begin assimilation for several days as judged by appearance of green colour in leaves and stems. After 7–10 days, assimilation appeared to take place normally, but the initial lag caused the plants to contain less N at harvest than did the others so that the calculated values of *g* for these two cultures were definitely lower.

Exp. 7. In this experiment further evidence of the occurrence of a lag in uptake of combined N by red clover plants previously kept in an atmosphere

Table III. *Effect of change in the atmosphere on assimilation of free and combined N (Exp. 7)*

	Harvest I to II			Harvest II to IV		Data of harvest III			Data of harvest IV		
Treatment	Total N mg.	% N	g*	Atmosphere	Source of N	Total N mg.	% N	g	Total N mg.	% N	g
Exp. 7:											
Air	13·1	2·64	0·075	Hydrogen†	N_2	21·4	1·90	0·047	—	—	—
						18·7	1·75	0·038	24·3	1·76	0·029
pH_2=0·6 atm.	4·63	1·46	0·040	Air	N_2	24·0	3·03	0·103	—	—	—
pN_2=0·2 atm.						22·0	3·14	0·097	38·8	2·75	0·075
pO_2=0·2 atm.				Air	NH_4	—	—	—	62·9	3·55	0·105
						30·3	3·93	0·118	44·0	3·76	0·054
				Air	NO_3	19·6	2·94	0·089	36·2	3·00	0·051
						31·2	3·43	0·120	40·0	3·49	0·065
				Hydrogen	NH_4	24·2	3·37	0·103	56·8	3·50	0·131
					.	20·8	2·79	0·093	42·4	3·52	0·091
				Hydrogen	NO_3	34·6	3·94	0·127	—	—	—
						25·2	3·29	0·106	46·8	3·41	0·064

* These values of *g* are for cultures kept in atmosphere indicated in column 1 throughout experiment (see Fig. 4).
† Hydrogen = pH_2, 0·6 atm.; pN_2, 0·2 atm.; pO_2, 0·2 atm.

All cultures planted and inoculated—19 August 1937.

Harvest I	Placed under atmospheres given in column 1—15 September.
II	Changed to treatment given in columns 5 and 6—5 October.
III	20 October.
IV	27 October.

Addition of combined N:

	$Ca(NO_3)_2$	$(NH_4)_2HPO_4$
6 October	24·2 mg.	24·2 mg.
12	24·2 mg.	24·2 mg.
20	24·2 mg.	24·2 mg.

containing H_2 was obtained. Two harvests were made following the change of treatment, but since the rates of fixation were not always constant, values of g were calculated for each harvest. The results, summarized in Table III, confirm those of the previous experiments. If inoculated red clover plants which have been grown in air until N fixation is actively under way are transferred to an atmosphere containing a pH_2 of 0·6 atm., there is an immediate decrease in the rate of fixation. On the other hand, if the plants are first kept in the H_2-containing atmosphere until a carbohydrate excess accumulates, and then placed in air, decided acceleration in the rate of N fixation occurs. But if such plants are furnished with sufficient combined N to stop fixation of the free element, uptake of the combined forms may be delayed for several days. After a lag period of 7 to 10 days assimilation of combined N begins, and the plants develop rapidly. They do not, however, succeed in overtaking those cultures which begin the assimilation of combined N immediately after its addition.

The cause of this unexpected lag in the uptake of combined N in some of the plant cultures is unknown, but the following characteristics of its occurrence should be noted.

(1) The lag is observed only in plants which have an excess of carbohydrate as a result of being placed in an atmosphere which restricts fixation of elemental N_2. In some respects its occurrence is the reverse of that previously noted by Fred et al. [1938] who found that soy beans high in carbohydrates began assimilation of free N_2 only after addition of small quantities of combined forms of this element.

(2) It is noteworthy that fixation of elemental N_2 by these clover plants was unaffected by the previous growth in the H_2-containing atmosphere. This difference in the assimilation of free and combined N might be used as support for the view of Virtanen & v. Hausen [1931] that the element is a superior source of N for nutrition of the clover plant.

(3) The lag period is not a typical response since, with the majority of the cultures, uptake of combined N occurred almost immediately after its addition, and certainly as soon as fixation of the free element could be detected in the inoculated cultures transferred to air.

(4) The delay in assimilation of combined N does not appear to be associated with the presence of H_2 since some of the plants transferred to air likewise exhibited the lag period.

DISCUSSION

The experiments reported in this paper together with those previously discussed [Wilson & Umbreit, 1937] provide four types of evidence for the view that H_2 is a specific inhibitor of N fixation by the symbiotic system in red clover. These are:

1. The total quantity of N fixed by inoculated red clover plants is linearly dependent on the pH_2 in the atmosphere, whereas the assimilation of combined N is independent of the pH_2 within experimental error.

2. Clover plants which have been grown in atmospheres of different partial pressures of H_2 show significant differences in the total quantity of elemental N_2 fixed, but similar plants supplied with combined N do not assimilate significantly different quantities of the combined forms.

3. The rates of assimilation of both free and combined N are essentially logarithmic during the period of growth under study which allows calculation of the unimolecular constant of N uptake—the so-called g value. Values of g are significantly different for plants grown in atmospheres of differing pH_2 only if the plants must use free N_2 for their source of this element.

4. Inhibition by H_2 is obtained at different stages of growth and is reversible. For example, inoculated clover plants transferred from an atmosphere containing H_2 to air immediately show an increase in the rate of fixation. Moreover, if the

plants are not transferred but supplied instead with combined forms of the element, uptake of the combined N and development of plants proceed at rates which are strictly comparable with those of similar plants kept in air.

Seventeen experiments have been made during 5 years with consequent fairly large variations in the environmental factors which will influence assimilation of either free or combined N, e.g. intensity and duration of light, temperature etc. The results of all the experiments consistently point to the view that the action of H_2 on the development of inoculated red clover plants is specifically associated with N fixation. All attempts to detect a significant effect on the general development of the plant apart from the fixation of free N_2 or to correlate the action with any particular stage or rate of growth were unsuccessful. On the basis of these data, it is therefore concluded that H_2 is a specific inhibitor for the symbiotic N-fixing system in red clover.

SUMMARY

Additional evidence has been furnished which confirms the conclusion reached from results of previous work: molecular H_2 is a specific inhibitor for the symbiotic N fixation process in inoculated red clover plants.

The assimilation of both free and combined N by red clover under the conditions of the reported experiments is sufficiently close to logarithmic to allow calculation of the unimolecular constants of N assimilation, the g values. These constants are particularly useful for detection of stimulating or inhibiting effects of a substance on the rate of a given reaction. Values of g for assimilation of free N_2 were significantly decreased through addition of H_2 to the atmosphere, but those for assimilation of combined N were independent of the presence of H_2 in the atmosphere.

That the inhibitory action of H_2 is not associated with any particular stage or rate of growth was demonstrated by transferring plants to an atmosphere containing H_2 after growing in air for various periods of time. It was also shown that the action was reversible since plants kept in the presence of H_2 immediately increased their rate of N assimilation if transferred to air or if combined N was supplied to them.

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CCLXX. THE ORIGIN AND SIGNIFICANCE OF SALIVARY PHOSPHATASE

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PART I. THE ORIGIN OF SALIVARY PHOSPHATASE

THE occurrence of a phosphatase in saliva is generally accepted. Demuth [1925] appears to have been the first to study the activity of this enzyme in saliva. Adamson [1929] and Smith [1930] made important advances in this subject. Both observers considered the phosphatase activity of saliva to be related to the formation of calculus (tartar) on the teeth.

It is desirable to consider the origin of salivary phosphatase. Adamson [1929] found that tissue removed from the gums showed phosphatase activity and that saliva contained a substrate for this enzyme, since on incubating saliva and this tissue (or bone phosphatase) there was an increase in free phosphate. He definitely associated the occurrence of these two factors with the formation of calculus on teeth, a process he was able to imitate *in vitro*. Smith [1930] followed up Adamson's work and found in saliva a phosphatase which he believed to originate from epithelial scales, because centrifuged saliva had no phosphatase activity. He found that this enzyme had an optimum pH of 5·5 and could hydrolyse the naturally occurring phosphoric esters of saliva as well as the Na glycerophosphate. He also found that if tissue were removed from the gums and placed in Ca glycerophosphate, a deposition of Ca phosphate occurred in the epithelium.

Since phosphatases are widely distributed in living matter and are found in many animal tissues, organs and secretions, their presence in saliva was readily accepted. Umeno [1931], however, found that neither saliva nor gastric juice contained any phosphatase, while both salivary glands and gastric mucosa showed phosphatase activity, although much less than kidney and duodenal mucosa. It occurred to us that since saliva contains a large variety of micro-organisms, these might be responsible, in part at least, for the phosphatase activity. Evidence is gradually accumulating that phosphatases occur commonly and perhaps generally in micro-organisms. Their occurrence in yeast juice is classical knowledge. The comparison of yeast and muscle metabolisms led to the association of phosphatases with carbohydrate breakdown. The phosphatases of takadiastase from *Aspergillus oryzae* have been studied systematically by Akamatzu and his colleagues, an account of their work being given in a review by Folley & Kay [1936]. Gordon & Cooper [1931; 1932] appear to have made the first observations on bacterial phosphatases. Heard & Wynne [1933] and Pett & Wynne [1933] studied the occurrence and properties of several types of bacteria of widely different cultural characteristics. Wiggert & Werkman [1938] remark that there is now considerable evidence for the importance of phosphorylation in the metabolism of micro-organisms.

We were able to demonstrate phosphatase activity in some micro-organisms, which are commonly air-borne and are therefore likely to be present in the mouth, and in two strains of actinomyces which are of special interest in the

present work, one freshly isolated from the human mouth. The full details of this part of the work will be given later.

To investigate the question of the origin of the salivary phosphatase, we set out to test the activity of cell-free and bacteria-free saliva. The phosphatase activity was tested in the following way, using 15 ml. graduated tubes.

Tubes 1 and 2. 10 ml. substrate + 5 ml. buffer pH 5·5 + 1 ml. chloroform water + 0·5 ml. saliva. Mix and add 2 ml. glacial acetic acid and put on ice. These are controls.

Tubes 3 and 4. As above, but incubate a suitable time before the addition of acid.

The volumes were all made up to 15 ml., filtered and the free phosphate estimated by the method of Fiske & Subbarow [1925]. Glacial acetic acid gives with saliva a much clearer filtrate than the more usually employed trichloroacetic acid, and in this way we avoided, in part, the difficulty of turbidity encountered by Smith. The excess of free phosphate in tubes 3 and 4 over that in tubes 1 and 2 gave the phosphatase activity of the saliva.

We compared the activities of native and centrifuged saliva (centrifuged at 2000 r.p.m. for 20 min.) and although the latter had less activity, it was by no means devoid of activity as Smith [1930] stated. Giri [1936] centrifuged saliva, in the process of purification of the phosphatase, without apparent loss of the enzyme. He was not, however, concerned with the origin of this enzyme. Saliva, filtered through nos. 30 and 42 Whatman filter papers, which keep back epithelial scales, also possessed phosphatase activity and, as expected, showed the presence of micro-organisms on culture and incubation in broth at 37° for 24 hr. Three samples of saliva which had been put through a Seitz filter (disk EK) and one which had been through a Berkefeld N filter were also investigated. The Seitz-filtered sample proved sterile, on testing by seeding into broth and incubating at 37°. The Berkefeld sample was not sterile.

These results are represented in Table I. It is to be noted that salivas show very different degrees of phosphatase activity and occasionally no activity. This, in itself, may be significant.

Table I. *Phosphatase activity of saliva. Hydrolysis of Na α-glycerophosphate at pH 5·5*

Sample	Native	Centrifuged	Filtered	Remarks
1	+ +	+	O	Seitz EK sample had no ptyalin and no Ca or P. Sterile
2	+ +	+	O	Ditto
3	O	–	–	Not pooled. Ptyalin present
4	+ +	+	S	Berkefeld. Not sterile
5	+ +	+	O	Seitz as above
6	O	–	–	Not pooled. Ptyalin present
7	+ +	+	–	

(S = slight activity; O = no activity; +, + + = activity of different degrees; – = not tested.)

In three of the experiments, the relative activities of native and centrifuged salivas were calculated, with the following results:

Saliva sample no.	4	5	7
$\dfrac{\text{Activity native}}{\text{Activity centrifuged}}$	1·4	1·4	3·1

The actual values obtained for sample 7 were:

Native saliva ... ... 30·0 mg. P set free in 18 hr. per 100 ml. saliva

Centrifuged saliva ... 9·5 mg. P set free in 18 hr. per 100 ml. saliva

In considering the filtered salivas, although the Seitz-filtered sterile saliva had no phosphatase activity and the Berkefeld-filtered non-sterile sample did possess activity, we cannot say, without reservation, that the activity of saliva is normally due to these organisms. An interesting observation was made in these saliva experiments. The Seitz-filtered saliva (a sample of the original had been shown to have all the normal constituents) contained no phosphatase and incidentally no ptyalin (amylase) and no inorganic P. The Berkefeld-filtered saliva had all the normal constituents.

Presuming that cannulated saliva collected under sterile conditions might be free from micro-organisms, two experiments were done on cats under chloralose.[1] The submaxillary duct was cannulated with a sterile cannula. Pilocarpine was injected intravenously to excite secretion. This duct secretion and the saliva from the mouth were tested for phosphatase activity. The results are given in Table II.

Table II. *Phosphatase activity of saliva*

	Substrate		
	Na α-glycero-phosphate 18 hr.	Di-Na-phenyl phosphate $\frac{1}{2}$ hr.	Remarks
Cat I:			
Duct secretion	S	O	Saliva very watery. Sterility not tested
Saliva from mouth	+ + +	O	Very viscid
Cat II:			
Duct secretion	S	O	Very watery. No cells present. Few bacteria in tubes incubated 18 hr.
Same duct secretion, mouth scrapings added	+ +	–	Scrapings from roof of mouth added
Saliva from mouth	+ + + + + +	+ +	Viscid. Cells and many organisms present

Note. The activity of saliva towards phenyl phosphate is always less than towards glycerophosphate, probably because at the optimum pH for salivary phosphatase, the phenyl phosphate does not hydrolyse well.

It is possible that more rigidly sterile conditions than we were able to obtain would give a secretion free of phosphatase. We do not think that the slight activity of the duct secretion was due to the presence of cells. Another possibility is that the other salivary or the buccal glands might produce the phosphatase.

If the desquamated cells in the saliva are not the sole source of the phosphatase, and it is thought that micro-organisms contribute to the activity, then clearly another way of investigating this latter possibility is to compare the phosphatase activity of saliva straight from the mouth, as uncontaminated with air-borne organisms as possible, with the activity of the same saliva incubated in a nutrient medium for a suitable time at 37°.

A series of such experiments was carried out, the saliva being tested as described previously at pH 6 using glycine buffer and glycerophosphate. The details of the conditions of these experiments and the results are set out in Table III.

There can be no doubt that saliva contains micro-organisms whose multiplication is accompanied by an increase in phosphatase activity. It may be noted here that Smith [1930] found that saliva had less activity after brushing

[1] These experiments were performed at Bedford College by M. M. Murray.

Table III. *Phosphatase activity of saliva after incubation*

		Phosphatase activity in mg. P/100 ml. saliva	
Details of incubation	Time of incubation in days	α-glycero-phosphate	β-glycero-phosphate
Saliva and Czapek-Dox (KH$_2$PO$_4$ omitted)	0	1·0	1·0
	1	1·0	1·0
	6	44	34
Saliva and sterile nutrient broth	0	4·6	—
	2	67	—
Saliva and sterile glucose broth	0	2·5	—
	3	58	—
The same in presence of 0·7 % T.C.P.	3	6·6	···

the teeth, a process which certainly diminishes the vegetative growth which forms part of dental "plaque". The conclusion we have arrived at from the experiments on centrifuged saliva, submaxillary duct saliva of the cat and the incubated saliva is that the phosphatase of saliva could originate, partly at least, from the micro-organisms present.

PART II. THE RELATION OF CALCULUS (TARTAR) FORMATION TO MICRO-ORGANISMS AND SALIVARY PHOSPHATASE

The work of Adamson [1929] and that of Smith [1930] lead to the same general conclusions, namely, that the deposition of Ca phosphate and hence also of calculus was brought about by the activity of the phosphatases in either gum tissue or saliva. Tenenbaum & Karshan [1937] found significantly higher Ca and P values in saliva from mouths where calculus formation was greater.

Calculus formation has been correlated with the occurrence of certain micro-organisms, viz. *Leptothrix* (now called *Actinomyces*) by several investigators and some of these workers, i.e. Goodrich & Moseley [1916] and Siegel [1935] relied on histological methods. Naeslund [1926] and Bibby [1935] used both cultural and histological methods, while Bulleid [1925] carried out experiments both *in vitro* and on cats from which he deduced that calculus formation was related to the occurrence of *Leptothrix*. It may be pointed out that Henrici [1930] cast doubt on the value of examination and identification of this type of organism when carried out solely by microscopical examination of stained preparations. This criticism of Henrici gains weight when the results just mentioned are contrasted with those of Lord & Trevett [1936] who obtained four cultures of *Actinomyces* from 90 mouths and Erikson [1937] who was only once successful in growing *Actinomyces* from teeth, although a number of attempts were made and the investigator had grown 50 strains of *Actinomyces* from various sources over a period of years [Erikson, 1935]. Attempts by one of us (P. P.) to isolate *Actinomyces* were at first unsuccessful even from a case of true clinical actinomycosis. Later in the progress of the work an aerobic *Actinomyces*, identical with those placed in group I by Erikson (after Ørskov), was isolated in pure culture from the mouth and this strain and also *A. buccalis* (Winslow), which had been maintained in a series of sub-cultures, were included in the study of the phosphatases of certain mouth micro-organisms. This latter culture was obtained from the Lister Institute (National Collection of Type Cultures) through the courtesy of Dr St John Brooks. It originally came from the American National Type Culture, having in the first place been isolated from the mouth.

Following up the line of thought that the phosphatase of saliva is possibly derived from micro-organisms, we were led to investigate in the early part of this work the phosphatase activities of certain micro-organisms which were considered to be common inhabitants of the mouth. In view of the fact that they are widely distributed in air, soil and food, there is no doubt that they are frequently present in the mouth, although they are not necessarily colonized there. These micro-organisms, originally obtained from a collection of extracted teeth and isolated in pure culture in an unorthodox manner, were first studied. Our attention was accidentally directed to these organisms in the process of separation and collection of enamel protein. Sound (non-carious) human incisor teeth were scraped, the roots cut off and the pulps removed. The crowns were exposed to 10 % KOH for periods up to ½ hr. and then brushed vigorously in order to remove the mucin. These crowns were washed well in water and then exposed to a large volume of 2 % HCl for about 48 hr. As decalcification proceeded, the enamel protein separated as small flakes and the dentine became soft. The dentine remnants were then picked out, leaving the few mg. of enamel protein in the form of floating fragments which could be collected by centrifuging. Work was then suspended for about 6 weeks, during which time the protein was left covered in 2 % acid at room temperature. It was hoped that bacterial growth would be restricted in this way, so that work of a bacteriological nature could be carried out on the protein. The pH of the liquid was about 0·7. At the end of the period mentioned, it was noted that some "fluffy" growth was attached to the protein. The sources of nitrogen available to the growth were very limited. It was thought at one time that the organisms were using enamel protein as the source of nitrogen, but although there were some indications that this may be true, we were not successful in showing histologically that these organisms had penetrated the pieces of protein. Further work on this point is in progress. From the "fluffy" mass three organisms were isolated. They have been kept in a series of sub-cultures for about 6 months and are pure strains. The method of isolation described above has most certainly eliminated many of the mouth organisms. We have, however, limited our investigations to some of those from human mouths which could withstand exposure to 2 % HCl for 6 weeks, and whose nitrogen requirements were limited to the very small amount of extraneous nitrogen-containing compounds which had not been eliminated.

Description of the organisms studied

The three organisms were originally described as "green", "red" and "white", but they have subsequently been classified as far as possible. The "green" organism is a typical strain of *Penicillium spinulosum* (Thom.). The "red" organism is a species of *Rhodotorula* resembling *Rhodotorula rubra* [Harrison, 1928]. Some difficulty has been met in identifying the "white" organism. It grows aerobically only, colonies on Czapek Dox, serum, agar and glucose-agar being smooth, shiny, raised, slowly spreading and very mucilaginous. In slide cultures the mycelium is non-septate. Spores have not been seen. It is thought that the "white" organism is one of the monilia. (The description of the two strains of *Actinomyces* studied has already been given.)

The method of isolation of the organisms from extracted teeth certainly eliminated many of the mouth organisms, but doubt remained as to whether the organisms studied came from the mouth. The teeth examined were at first collected in water saturated with chloroform in small jars, when some moulds grew on the corks. Later all corks and jars were sterilized by exposure to hot

air at 160° for 1 hr., a method which proved to be satisfactory. In some collections of teeth made in these sterilized jars, penicillia still grew. Mould spores from the air might have contaminated the teeth in the interval of 2–4 hr. between the removal from the mouth and the placing in the jars. This possibility was not investigated.

It is to be noted that the primary object of the study of these organisms was to learn something of their proteolytic activity. Simultaneously, however, partly at the suggestion of Prof. F. C. Wilkinson, the possibility of the occurrence of phosphatase came under consideration.

Study of phosphatase activity

Three esters were used as substrates, namely Na α- and β-glycerophosphates (Boots) and di-Na-phenyl phosphate (B.D.H.), the latter purified to remove free phosphate. Each ester was tested at the characteristic pH optima of acid and alkaline phosphatases [Folley & Kay, 1936], i.e. at pH 3·8–4·0 and pH 9·9, using Sørensen's glycine and HCl and glycine and NaOH buffers [Clark, 1928], and also at pH 5·5, which was shown to be the optimum of salivary phosphatase by Smith [1930].

The enzyme preparations were made by scraping the growth (4–7 days old) off a Petri dish (10 cm. diameter) agar culture and grinding with sand and sufficient volume of 0·7 % $MgSO_4$ saturated with chloroform to give enough solution for the tests. This was filtered through cotton-wool. One ml. of such a solution contains the optimal concentration of Mg for phosphatase activation. The substrates were 0·1 % Na α- and β-glycerophosphates and 0·109 % di-Na-phenyl phosphate. The tests and adequate controls were carried out according to the following directions. Twelve tubes were set up for each growth with each substrate, each tube containing 10 ml. of the particular substrate, 5 ml. of one of the buffer solutions and 1 ml. of the growth extract. Half of these tubes served as controls and were left in the refrigerator after the addition of 2 ml. 25 % trichloroacetic acid, to stop any enzymic action. The other tubes were incubated at 37° in the case of the phenyl phosphate for ½ hr. and in the case of the glycerophosphates for 18 hr. After this incubation time, the enzyme activity was stopped in the same way as the controls.

Tubes 1 and 2. 10 ml. substrate, 5 ml. buffer pH 4 and 1 ml. extract. Incubated at 37° before the addition of acid.

Tubes 3 and 4. 10 ml. substrate, 5 ml. buffer pH 4 and 1 ml. extract. 2 ml. trichloroacetic acid added and tubes put on ice.

Tubes 5 and 6. 10 ml. substrate, 5 ml. buffer pH 5·5 and 1 ml. extract. Incubated before addition of acid.

Tubes 7 and 8. 10 ml. substrate, 5 ml. buffer 5·5 and 1 ml. extract. Acid added as above and put on ice.

Tubes 9 and 10. 10 ml. substrate, 5 ml. buffer pH 10, 1 ml. extract. Incubated before addition of acid.

Tubes 11 and 12. 10 ml. substrate, 5 ml. buffer pH 10, 1 ml. extract. Acid added as above and put on ice.

All the tubes were then brought to room temperature and filtered. 10 ml. of each filtrate were tested for free P by the method of Fiske & Subbarow [1925].

Successive sub-culturings were tested in this way and although the results (Table IV) with each of the cultures are not quantitatively comparable, the results obtained with any one culture at different pH in any one series are strictly comparable.

Table IV. *The phosphatase activities of the micro-organisms on different substrates at different pH*

Substrate pH	Na α-glycero-phosphate			Na β-glycero-phosphate			Di-Na-phenyl-phosphate		
	4	5·5	10	4	5·5	10	4	5·5	10
Monilia (white growth)	O	S	+ +	O	S	+ +	O	+	+ + +
R. rubra (Harrison) (red growth)	O	+	S	O	+ +	+	O	S	+ + +
P. spinulosum (Thom.) (green growth)	+	+ + +	S to O	S to O	+ + +	S	O	+ + + +	+
Mixed white and green	O	+ +	O	O	+ +	O	O	+ +	O
A. buccalis (Winslow)	O	O to S	O	-	-	-	-	-	-
Actinomyces, group 1, Erikson	S	+ + +	S	-	-	-	-	-	-
Saliva	+	+ + +	S	O	+ +	O	O	S	O

In our choice of *p*H values at which to test the enzyme activity, we had in mind the separation of the acid and alkaline phosphatases [Folley & Kay, 1936] and the optimum reaction for the salivary phosphatase towards glycerophosphate found by Smith [1930] to be *p*H 5·5, by Giri [1936] *p*H 5·0 and by us *p*H 6·0.

It can be seen from Table IV that the different growths must have different *p*H optima. We were most interested in those showing greatest activity at *p*H 5·5–6 and hence further investigations were made with the green growth. In spite of the fact that *A. buccalis* (Winslow) was tested in six separate young sub-cultures at *p*H 5·5 and *p*H 10, on three different substrates, we were only able to show very slight phosphatase activity and this not constantly. This result must be contrasted with the marked activity of the *Actinomyces* (group 1) freshly isolated from the human mouth. It is quite possible that *A. buccalis* (Winslow) when first isolated, had greater activity. Henrici [1930] noted that *A. gypsoides*, although actively proteolytic when first isolated, gradually, and eventually completely, lost this power after some months of subculturing.

The tests on *Actinomyces* (group 1) were carried out at *p*H 6 only, since it had been shown to have a broad optimum between *p*H 6 and 7.

It is concluded from these tests that certain mouth organisms, including *Actinomyces*, can exhibit phosphatase activity in the mouth. It is, of course, possible that many other mouth organisms also possess phosphatase activity [Fosdick, 1937]. Incidentally, it may be noted that the caries research group of the North Western University, Chicago [see Hatton, 1938], are of the opinion that caries is possibly the result of a combined action of micro-organisms having phosphatase activity, possibly yeasts, together with acid-producing bacteria. We do not wish to comment on this idea, since we have done no work on this point. If, however, the occurrence of these phosphatases is significant, we consider that they should hydrolyse the ester P of blood and saliva.

The green growth was tested on a neutralized trichloroacetic acid protein-free filtrate of whole blood, rat and human, in a similar manner to that of Byrom & Kay [1928] to show that part of the ester P of blood is hydrolysable by kidney phosphatase. An increase of free P resulted on incubation of the green growth with these blood filtrates. With the human blood, the increase amounted to 15 mg. P per 100 ml. blood, a degree of hydrolysis which suggests that the culture had several types of phosphatase in it.

Wilkinson [1935] put forward histological evidence that calculus deposition could be subgingival where no breakaway of the soft tissues from the teeth had occurred. We remark here that these micro-organisms could use the organic

hydrolysable P of the blood plasma and so increase the concentration of free PO_4, which, together with the Ca of the plasma or saliva could conceivably lead to the deposition of Ca phosphate.

It was also considered necessary to show that the saliva itself contains a substrate for these phosphatases. For this test, centrifuged saliva was steamed for 5 hr. without concentration. It became cloudy, but was used without further treatment as a substrate for the green mould in the following experiment:

Tubes 1 and 2. 5 ml. saliva (steamed), 5 ml. buffer pH 5·5, 2 ml. glacial acetic acid. Placed on ice.

Tubes 3 and 4. As tubes 1 and 2, but incubated for 18 hr. at 37° before addition of the acid.

Tubes 5 and 6. 5 ml. water to replace the saliva, otherwise the same as tubes 1 and 2.

Tubes 7 and 8. 5 ml. water to replace saliva, otherwise the same as tubes 3 and 4.

This experiment yielded a positive result. Tubes 3 and 4 had greater free PO_4 content than tubes 1 and 2. The actual estimation of this PO_4 set free from the saliva was 0·93 mg. hydrolysable P per 100 ml. saliva. Smith [1930] found between 0·8 and 2·7 mg. organic P per 100 ml. saliva. We consider we have demonstrated the fact that green mould can use saliva as a substrate. Furthermore, extracts of the green *Penicillium* incubated with a 1 % solution of Ca glycerophosphate, led to the deposition of a white precipitate, which was readily soluble in dilute acid and was found to contain both Ca and P.

In showing that common mouth organisms, including *Actinomyces*, contain phosphatases which can use serum or saliva as a substrate, a correlation has been brought about between the observations of Adamson [1929] and Smith [1930] on the one hand, and Naeslund [1926] and Bulleid [1925] and Bibby [1935] on the other, concerning the theories of calculus formation.

SUMMARY

1. Human saliva exhibits variable phosphatase activity which is maximum towards glycerophosphate at pH 5·0–6·0.

2. The submaxillary duct secretion of cats had only very slight phosphatase activity as compared with the mouth saliva of the same animals at the same time.

3. The phosphatase activity of human saliva is not entirely due to the epithelial scales.

4. Certain common mouth micro-organisms exhibit marked phosphatase activity.

5. The organisms studied included *Penicillium spinulosum* (Thom), *Rhodotorula rubra* (Harrison), a monilia, *Actinomyces buccalis* (Winslow) and *Actinomyces* (group 1, Erikson).

6. The *Penicillium* was able to use the hydrolysable ester phosphorus of blood and saliva as a substrate.

7. Precipitation of calcium phosphate occurs when calcium glycerophosphate is incubated with the *Penicillium*. This may be analogous to calculus formation.

8. The correlation of phosphatase activity with the presence of micro-organisms, especially *Actinomyces*, is important in relation to the theories of calculus formation which have been advanced previously.

For the identification of the *Penicillium* and the *Actinomyces* (group 1) **we** are indebted respectively to Mr G. Smith and Miss D. Erikson, both of the London School of Hygiene and Tropical Medicine. We wish to thank the Medical Research Council for grants towards the expenses of this work and for personal grants to two of the workers (P. P. and G. E. G.).

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CCLXXI. ENZYMIC PROTEOLYSIS

III. HYDROLYSIS OF ASPARAGINE, ASPARAGINE PEPTIDES AND ANHYDROGLYCYL-ASPARAGINE

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THE experiments to be described in the present paper are complementary to those published in a previous communication [Damodaran & Ananta-Narayanan, 1938] on the formation of ammonia during the enzymic hydrolysis of natural proteins. Bergell & Wulfing [1910], who seem to have been the first to study the deamidizing action of proteolytic enzymes on synthetic substrates, showed that *dl*-leucinamide is hydrolysed by pancreatin, the *l*-compound being more readily decomposed than the *d*-isomeride; negative results were recorded in the case of glycinamide, alaninamide and natural asparagine. According to Levene *et al.* [1926] "filtered intestinal juice" splits glycinamide as readily as alanylalanine though not with the same ease as glycylglycine. Grover & Chibnall [1927] observed the hydrolysis of asparagine by intestinal erepsin as well as by a dipeptidase preparation obtained from germinating barley. The hydrolysis of glycinamide and leucinamide was confirmed by Waldschmidt-Leitz *et al.* [1927] using trypsin-free erepsin prepared by adsorption. Melville [1935] attempted to study the deamidizing action of proteolytic enzymes on γ-glutaminyl peptides, but found that the substrates were so labile that they were spontaneously decomposed in aqueous solutions at the pH required for enzyme action.

In the present investigation we have studied the actions of pepsin, trypsin, erepsin and papain on asparagine, glycinamide, *dl*-leucinamide, glycyl-*l*-asparagine, *d*- and *l*-leucylasparagine, chloroacetyl-*l*-asparagine and anhydro-glycylasparagine (2:5-diketopiperazine acetamide). Amide hydrolysis was measured by actual distillation of the ammonia and estimation of the latter by a sensitive micro-method. To ensure that quantitative differences in the rate of splitting were not interpreted to mean qualitative differences in specificity, enzyme action was in all cases allowed to proceed for fairly long periods. Some of the substrates, particularly glycinamide, glycyl- and leucyl-asparagine, were found to be hydrolysed to a slight extent at the pH employed for enzyme action, but rigorous controls were run to determine the exact extent of such spontaneous decomposition. The results are summarized in Table I.

Pepsin and purified trypsin have no action on either the peptide or amide bonds in any of the substrates examined. Trypsin-free erepsin on the other hand has a definite, though slow, deamidizing action on asparagine, the hydrolysis of the amide group proceeding more rapidly in fact than in glycinamide though less rapidly than in leucinamide. The amide group in asparagine peptides does not appear to be directly hydrolysed; a small amount of ammonia is formed from glycylasparagine and *l*-leucyl-*l*-asparagine, but this must depend on the preliminary hydrolysis of the peptide to give free asparagine as no ammonia liberation takes place from *d*-leucylasparagine in which the peptide bond is not split by erepsin. Of particular interest in connexion with peptide hydrolysis is the finding that in chloroacetylasparagine the peptide bond is split by erepsin in

Table I. *Hydrolysis* %

	Trypsin-free erepsin		Erepsin-free trypsin		Pepsin		Papain-HCN	
	Peptide	Amide	Peptide	Amide	Peptide	Amide	Peptide	Amide
l-Asparagine	--	10 15 days	—	0·37 7 days	—	0·43 6 days	—	0·33 9 days
Glycinamide	—	10 10 days	—	Nil	—	2·9 9 days	—	1·45 8 days
dl-Leucinamide	—	53 10 days	—	1·0 7 days	—	Nil	—	11·0 7 days
Glycyl-*l*-asparagine	97 10 days	8 26 days	Nil	Nil	Nil	Nil	Nil	Nil
d- and *l*-Leucyl-*l*-asparagine	17 6 days	2 6 days	,,	,,	,,	,,	,,	,,
d-Leucyl-*l*-asparagine	Nil	Nil	,,	,,	,,	,,	,,	,,
Chloroacetyl-*l*-asparagine	77 10 days	10 10 days	,,	,,	,,	,,	,,	,,
Anhydroglycylasparagine	Nil	Nil	,,	,,	,,	,,	,,	,,

spite of the absence of a free amino group adjacent to it; this is analogous with the hydrolysis by this enzyme of chloroacetylalanine observed by Abderhalden & Ehrenwall [1930]. According to the views on enzymic specificity developed by Waldschmidt-Leitz, Grassmann and collaborators (summaries by Grassmann [1928, 1932, 1936]) trypsin-free erepsin consists of a mixture of aminopolypeptidase and dipeptidase. The observations now recorded can be brought into line with the views on enzyme specificity developed by the Willstätter school only on the assumption that, in addition to di- and aminopoly-peptidases, erepsin also contains asparaginase and other amidases as well as peptidases that act specifically on acyl peptides of the type of chloroacetylasparagine. However, the weight of available evidence is against the acceptance of these views of the mode of action of the proteases as final. On the one hand the identification of some of the proteolytic enzymes with crystalline proteins [Northrop, 1930; Northrop & Kunitz, 1934], the changes in specificity shown to take place on keeping of enzymes purified by adsorption [Abderhalden & Schmitz, 1929; Abderhalden & Ehrenwall, 1931] and the dependence of the range of activity of proteases on physical conditions [Fodor, 1930] make it highly improbable that the enzyme preparations with restricted activity obtained by Waldschmidt-Leitz, Grassmann and others really represent individual enzymes in the natural state and not merely fragments of such. Further, numerous recent studies on synthetic peptides, in particular by Abderhalden and by Bergmann, make it clear that the chemical bases of the specificity relations enunciated by the Willstätter school for their enzyme preparations will have to be considerably modified.

With papain-HCN only a slow deamidation with leucinamide and hardly any with other substrates was noticed. This appears to be in contradiction with the results obtained previously [Damodaran & Ananta-Narayanan, 1938] which showed that papain digestion led to the formation of considerable amounts of ammonia. It has, however, been recently shown by Bergmann *et al.* [1935] that the amide group in peptide amides in which there is no free amino group in the proximity of the amide group is attacked by papain.

The observation that anhydroglycylasparagine is not hydrolysed either at the peptide or the amide bond by any of the enzymes tried is contrary to the claims

of Japanese workers [cf. Shibata, 1934] who claim to have demonstrated the enzymic hydrolysis of diketopiperazines, and provides further confirmation of the findings of Waldschmidt-Leitz & Gärtner [1936] with diketopiperazine acetic and propionic acids and of Greenstein [1936] with lysylglutamic acid anhydride.

With reference to the problem in protein structure which primarily prompted this investigation, the results obtained, taken in conjunction with previous findings [Damodaran, 1932; Damodaran et al. 1932; Melville, 1935; Damodaran & Ananta-Narayanan, 1938], agree with the assumption that the amide groups attached to the dicarboxylic amino-acids are the only important precursors of the ammonia formed in the acid hydrolysis of proteins. In the last paper cited above it was concluded that the ammonia liberated during the digestion of proteins by pepsin, trypsin and erepsin in succession was not primarily connected with enzyme action. If asparagine and glutamine are the only ammonia-yielding complexes in proteins this is readily understandable for the following reasons. Peptides of glutamine formed through its free (γ) carboxyl group are, unlike those formed through the amino group studied by Thierfelder & Cramm [1919], as unstable as glutamine and spontaneously decompose in aqueous solution [Melville, 1935] giving rise to ammonia. This non-enzymic ammonia formation could commence practically at the very beginning of peptic digestion and would increase progressively with the amount of glutamine-containing polypeptides set free. Asparagine peptides on the other hand are quite stable in aqueous solution and also, as shown in this paper, not acted upon by either pepsin, trypsin or erepsin. The deamidizing action which erepsin has been shown to have on *free* asparagine can come into play only when digestion is practically complete and is at the same time too slow to cause any appreciable liberation of ammonia. When it is further considered that the proportion of aspartic acid in proteins is invariably much lower than that of glutamic acid it is obvious that practically the whole of the ammonia formed during digestion has its origin in the secondary non-enzymic decomposition of glutamine peptides and of glutamine.

EXPERIMENTAL

Substrates

Glycinamide hydrochloride and dl-*leucinamide* hydrobromide were prepared according to Bergell & Wulfing [1910].

Glycyl-l-*asparagine*. The purified peptide prepared by the method of Fischer & Koenigs [1904] browned at 215° and gave the reactions described by these authors. N found 22·07 %: calc. for $C_6H_{11}N_3O_4$, 22·22 %. Amino-N (Van Slyke) was 124 % of the theoretical, in keeping with the observed abnormality of glycine and glycyl polypeptides in general [Van Slyke, 1911; Fischer & Koelker, 1910].

Leucyl-l-*asparagine* [Fischer & Koenigs, 1907]. Asparagine was coupled with bromo*iso*hexoylbromide and the resulting compound treated with 75 % ammonia at room temperature for 6 days. Owing to the difference in solubility between the two isomeric bromo*iso*capronylasparagines noted by Fischer & Koenigs, two products were finally obtained, viz. pure d-leucyl-l-asparagine and a mixture of d- and l-leucyl-l-asparagines in which the compound containing the non-natural d-leucyl residue predominated. Attempts to obtain the pure l-leucylpeptide in pure condition by fractional crystallization were not successful. $C_{10}H_{19}N_3O_4$, $2H_2O$ requires N, 14·95 %; N, found, 15·2 % (for the d form) and 15·12 % (for the mixture of d- and l-).

Anhydroglycylasparagine. This was prepared according to Fischer & Koenigs [1904].

Enzymes

Erepsin-free trypsin [Waldschmidt-Leitz & Harteneck, 1925], trypsin-free erepsin [Waldschmidt-Leitz & Schäffner, 1926] and B.D.H. pepsin were used. Papain was prepared as previously described [Damodaran & Ananta-Narayanan, 1938].

Buffer solutions

For erepsin studies $M/5$ phosphate buffer at pH 7·8, for trypsin $M/5$ veronal buffer at pH 8·4 and for papain $M/10$ citrate buffer at pH 5 were employed. As the usual NH_4OH/NH_4Cl buffer for tryptic studies could not be used on account of the necessity of making ammonia determinations, experiments were conducted to ascertain the suitability of veronal buffer. It was found that veronal could effectively replace phosphate as well as NH_4OH/NH_4Cl in erepsin and trypsin digests. It has the advantage over the former of not interfering with titrations in alcohol or formalin.

Methods

Weighed amounts of the analytically pure subtrates were dissolved in water, neutralized with a few drops of NaOH and, after the addition of buffer solution and enzyme, made up to give usually $M/10$ solutions. A few drops of toluene were added as antiseptic.

Peptide hydrolysis was determined by the usual alcoholic titration and ammonia liberation according to Parnas & Heller [1924] with the modification that when the ammonia to be determined was small titration was carried out by the iodimetric method of Henriques & Christiansen [1916] using $N/280$ acid.

(1) *Asparagine*

Table II. *Exp. 1. Trypsin-free erepsin on* l-*asparagine*

Digest: $M/10$ asparagine containing 10 ml. of erepsin solution in total volume 25 ml. at pH 7·8 in phosphate buffer.

Ammonia as ml. $N/280$ acid in 2 ml.

Time	Digest	Substrate control	Enzyme control	Digest (corrected)	Amide hydrolysis %
0	0·04	Nil	0·04	Nil	Nil
6 hr.	0·20	Nil	0·03	0·17	0·32
18 hr.	0·79	Nil	0·03	0·76	1·36
42 hr.	1·92	0·02	0·06	1·85	3·30
4 days	4·00	0·09	0·08	3·82	6·80
10 days	8·78	0·18	0·08	8·52	15·20
19 days	12·16	0·34	0·13	11·65	20·80

Exp. 2. Erepsin-free trypsin on l-*asparagine.* Digest: $M/10$ asparagine containing 5 ml. of trypsin solution in total volume 25 ml. at pH 8·4 (veronal buffer).

Amide hydrolysis in 7 days, 0·37 %.

Exp. 3. B.D.H. pepsin on l-*asparagine.* Digest: 10 ml. of $M/4$ asparagine +5 ml. pepsin solution in $N/20$ HCl containing 1/20 the weight of dry pepsin calculated on the weight of the subtrate +5 ml. $N/5$ HCl. Total volume 25 ml.

Amide hydrolysis in 6 days, 0·43 %.

Exp. 4. Papain-HCN on l-*asparagine.* Digest: 10 ml. $M/4$ asparagine. 5 ml. papain solution 10 ml. citrate buffer pH 5 in total volume 25 ml. No NH_3 in substrate control.

Amide hydrolysis in 9 days, 0·33 %.

(2) Glycinamide

Table III. *Exp. 5. Trypsin-free erepsin on glycinamide*

Digest: $M/12$ glycinamide containing 10 ml. erepsin solution in total volume 25 ml. at pH 7·8 in phosphate buffer.

Ammonia as ml. $N/280$ acid in 1 ml.

Time	Digest	Substrate control	Enzyme control	Digest (corrected)	Amide hydrolysis %
0	0·16	0·12	0·04	0	0
5 hr.	0·26	0·12	0·04	0·10	0·43
21 hr.	0·55	0·25	0·03	0·27	1·16
46 hr.	0·93	0·35	0·08	0·50	2·15
4 days	1·65	0·59	0·06	1·00	4·29
10 days	3·11	0·70	0·08	2·33	10·00
19 days	4·48	1·69	0·11	2·68	11·50

Exp. 6. Erepsin-free trypsin on glycinamide. Digest: 0·3467 g. glycinamide HCl dissolved in water, neutralized with NaOH and made up to 25 ml. 10 ml. of this solution + 5 ml. veronal buffer at pH 8·4 + 5 ml. trypsin solution + water to 25 ml.

Amide hydrolysis in 7 days, nil.

Exp. 7. Pepsin on glycinamide. Digest: 0·1906 g. glycinamide HCl dissolved in sufficient HCl so that the final concentration of the HCl was $N/20$. 5 ml. of this solution removed and made up to 6·5 ml. for substrate control. To the residual 20 ml. 5 ml. pepsin solution in $N/20$ HCl added.

Amide hydrolysis, 1·1 % in 2 days, 1·9 % in 9 days.

Exp. 8. HCN-activated papain on glycinamide. Digest: 0·2241 g. glycinamide HCl + 10 ml. citrate buffer, pH 5, to total volume 25 ml. 5 ml. removed for substrate control. Residual 20 ml. + 5 ml. enzyme formed the digest.

Amide hydrolysis, 0·85 % in 65 hr., 1·45 % in 8 days.

(3) dl-Leucinamide

Table IV. *Exp. 9. Trypsin-free erepsin on dl-leucinamide*

Digest: 0·3517 g. *dl*-leucinamide HBr made up to 25 ml. after neutralization and addition of $M/5$ phosphate buffer pH 7·8. 10 ml. of this solution + 5 ml. erepsin made up to 25 ml. with water.

Ammonia as ml. $N/280$ acid in 2 ml.

Time	Digest	Substrate control	Enzyme control	Digest (corrected)	Amide hydrolysis %
0–¼ hr.	0·41	0·19	0	0·22	1·47
6 hr.	2·05	—	0	1·86	12·45
7 hr.	2·14	—	0	1·95	13·05
14 hr.	3·74	—	0	3·55	23·75
24 hr.	5·54	—	0	5·35	35·81
40 hr.	6·05	0·11	0	5·94	39·76
10 days	8·05	0·14	0	7·91	52·94
19 days	7·99	0·17	0	7·84	52·34

Exp. 10. Erepsin-free trypsin on dl-leucinamide. Digest: 0·175 g. *dl*-leucinamide HBr dissolved in water, neutralized and made up to 25 ml. with veronal buffer pH 8·4. 5 ml. of this removed for substrate control. The residual 20 ml. + 5 ml. trypsin formed the digest.

Amide hydrolysis in 7 days, 0·70 %.

Exp. 11. Pepsin on dl-*leucinamide.* Digest: 0·236 g. *dl*-leucinamide HBr dissolved in 25 ml. 5 ml. removed for substrate control. Residual 20 ml.+5 ml. pepsin formed the digest.

Amide hydrolysis in 8 days, nil.

Table V. *Exp. 12. Papain-HCN on* dl-*leucinamide*

Digest: 0·1902 g. *dl*-leucinamide HBr at *p*H 5 containing 5 ml. papain in total volume 25 ml.

	Ammonia as ml. $N/280$ acid in 2 ml.				Amide hydrolysis %
Time	Digest	Substrate control	Enzyme control	Digest (corrected)	
0	2·04	0	2·04	0	0
4 hr.	1·94	0	—	0	—
16 hr.	2·02	0	· ·	0	—
25 hr.	2·23	0	—	0·19	0·94
51 hr.	2·71	0	—	0·67	3·30
4 days	4·01	0	—	1·97	9·70
7 days	4·25	0	—	2·21	10·90

(4) Glycyl-l-asparagine

Table VI. *Exp. 13. Trypsin-free erepsin on* glycyl-l-*asparagine*

Digest: 0·5906 g. glycyl-*l*-asparagine neutralized and made up to 25 ml. with phosphate buffer of *p*H 7·8; 5 ml. of this solution removed for substrate control. Residual solution treated with 5 ml. of erepsin. The control showed no ammonia.

Time	Ammonia in 1 ml. of digestion as ml. $N/280$ acid	% amide split	ml. $N/10$ KOH for 2 ml. of digest	Increase in titre	% peptide split
0	0·18	0	3·68	0	0
4 hr.	0·22	0·14	4·38	0·7	35·2
13 hr.	0·64	1·64	4·99	1·31	65·6
18½ hr.	0·64	1·64	5·18	1·50	75·2
24 hr.	0·78	2·14	5·28	1·60	80·0
48 hr.	0·90	2·57	5·38	1·70	84·6
96 hr.	0·90	2·57	5·60	1·92	95·9
10 days	1·05	3·11	5·63	1·95	97·5
26 days	2·37	7·82	—	—	—

Exp. 14. Erepsin-free trypsin on glycyl-l-*asparagine.* Digest: 0·5906 g. of the substrate made up to 25 ml. with veronal buffer *p*H 8·4. 5 ml. removed for substrate control. Residual 20 ml.+5 ml. trypsin formed the digest.

No peptide or amide hydrolysis at the end of 5 days.

Exp. 15. Pepsin on glycyl-l-*asparagine.* Digest: 0·5906 g. of glycyl-*l*-asparagine dissolved in 5 ml. *M*/5 HCl and made up to 25 ml. 5 ml. of this removed for substrate control and diluted to 6·5 ml. with $N/20$ HCl. Residual 20 ml. of the solution+5 ml. pepsin formed the digest. No ammonia in enzyme controls.

No amide or peptide hydrolysis in 7 days.

Exp. 16. Papain-HCN on glycyl-l-*asparagine.* Digest: 0·463 g. substrate at *p*H 5 in citrate buffer containing 5 ml. papain-HCN solution in total volume 25 ml. No substrate control.

No amide or peptide hydrolysis in 9 days.

(5) d- and l-Leucyl-l-asparagines

Table VII. *Exp. 17. Trypsin-free erepsin on d- and l-leucyl-l-asparagines*

Digest: 1·405 g. of the substrate dissolved in water and neutralized. 10 ml. of erepsin made up to 25 ml. with phosphate buffer pH 7·8. Controls showed no change and are not included in the table.

Time	Ammonia as ml. $N/280$ acid in 1 ml. digest	% amide split	ml. $N/10$ KOH for 2 ml. digest	Increase in titre value	% peptide split
0	0·5	0	3·61	—	—
6 hr.	—	0	4·09	0·48	12·0
12 hr.	0·56	0·10	4·16	0·55	13·75
24 hr.	0·54	0·10	4·21	0·60	15·0
36 hr.	0·70	0·36	4·23	0·62	15·5
54 hr.	0·88	0·68	4·27	0·66	16·5
5 days	1·21	1·26	4·28	0·67	16·75
10 days	1·70	2·14	—	—	—

Exp. 18. Erepsin-free trypsin on d- and l-leucyl-l-asparagines. Digest: 0·620 g. of the substrate dissolved in water neutralized and made up to 25 ml. with 10 ml. veronal buffer pH 8·4 and 5 ml. trypsin. No controls.

No amide or peptide hydrolysis in 6 days.

Exp. 19. Pepsin on d- and l-leucyl-l-asparagines. Digest: 0·878 g. of substrate dissolved in 25 ml. of water and sufficient $N/20$ HCl to bring to pH 1·8. 5 ml. removed for substrate control. Residual solution + 5 ml. pepsin formed the digest. Enzyme control showed no change.

No amide or peptide hydrolysis in 8 days.

Exp. 20. Papain-HCN on d- and l-leucyl-l-asparagines. Digest: 0·502 g. of the peptide dissolved in water and made up to 25 ml. with 5 ml. papain solution and 10 ml. citrate buffer pH 5. No substrate control.

No amide or peptide hydrolysis at the end of 7 days.

(6) d-Leucyl-l-asparagine

Exp. 21. Trypsin-free erepsin on d-leucyl-l-asparagine. Digest: 1·00 g. of substrate dissolved in water, neutralized, and made up to 25 ml. with sufficient phosphate buffer at pH 7·8. 5 ml. removed for substrate control. To the residual 20 ml., 5 ml. erepsin added.

No peptide hydrolysis in 12 days: amide split 0·21 %.

Exp. 22. Erepsin-free trypsin on d-leucyl-l-asparagine. Digest: 0·6342 g. of peptide dissolved in water, neutralized and made up to 25 ml. with 5 ml. enzyme and 10 ml. veronal buffer of pH 8·4.

In 7 days, amide split 0·25 %, peptide split nil.

(7) Chloroacetyl-l-asparagine

Table VIII. *Exp. 23. Trypsin-free erepsin on chloroacetyl-l-asparagine*

Digest: 1·264 g. chloroacetyl-l-asparagine neutralized and made up to 25 ml. with phosphate buffer at pH 7·8. 10 ml. of this + 10 ml. erepsin diluted to 25 ml. formed the digest. Substrate and enzyme controls showed no change.

Time	Ammonia as ml. $N/280$ acid in 2 ml. digest	% amide split	ml. $N/10$ KOH for 2 ml. digest	% peptide split
0	0·09	—	1·43	—
6 hr.	0·15	0·22	1·65	11·3
18 hr.	0·18	0·33	2·01	29·9
24 hr.	0·24	0·55	2·04	31·5
48 hr.	0·73	2·69	2·07	33·0
96 hr.	1·40	5·15	—	—
6 days	—	—	2·92	76·8
10 days	2·76	10·20	—	—

Exp. 24. Erepsin-free trypsin on chloroacetyl-1-asparagine. Digest: 0·6515 g. substrate neutralized and made up to 25 ml. with veronal buffer *p*H 8·4. 5 ml. removed for substrate control. Residual 20 ml. + 5 ml. trypsin formed the digest.

In 7 days, amide split 0·2 %, peptide split nil.

(8) *Anhydroglycylasparagine*

Exp. 25. Trypsin-free erepsin on anhydroglycylasparagine. Digest: 0·7944 g. dissolved in 10 ml. phosphate buffer *p*H 7·8. 2 ml. removed for substrate control. Residue made up to 25 ml. with 10 ml. erepsin + water.

No amide or peptide split at the end of 6 days.

Exp. 26. Erepsin-free trypsin on anhydroglycylasparagine. Digest: 0·3086 g. substrate in 10 ml. water. 2 ml. removed for substrate control and the residue made up to 25 ml. with 5 ml. trypsin and 10 ml. buffer.

No amide or peptide split at the end of 7 days.

Exp. 27. Pepsin on anhydroglycylasparagine. Digest: the solution in Exp. 25 was used after inactivation of erepsin, adjustment of *p*H to 1·8 and addition of pepsin. The final strength of the solution was 0·041 N.

No amide or peptide split in 4 days.

Exp. 28. Papain-HCN on anhydroglycylasparagine. Digest: 0·2856 g. anhydroglycylasparagine dissolved in 10 ml. 2 ml. removed for substrate control and the residue diluted to 25 ml. after addition of 10 ml. citrate buffer *p*H 5 and 5 ml. papain solution.

No amide or peptide split at the end of 7 days.

SUMMARY

1. The amide group in leucinamide, asparagine and glycinamide is hydrolysed by trypsin-free erepsin, the rate of hydrolysis decreasing with the three substrates in the order given.

2. Trypsin-free erepsin also hydrolyses the peptide group in chloroacetylasparagine, but not the amide group in either this or other asparagine peptides examined.

3. Pepsin, erepsin-free trypsin and papain are without action upon the amide or peptide groups in glycinamide, leucinamide, asparagine, glycylasparagine or leucylasparagine.

4. Anhydroglycylasparagine (2:5-diketopiperazine acetamide) is not affected by any of the animal proteases or by papain.

5. The bearing of these results on (i) the specificity of the proteolytic enzymes, and (ii) the "amide" nitrogen of proteins is briefly discussed.

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CCLXXII. PREPARATION OF SOME PROTEIN SAMPLES FROM THE FRESH LEAVES OF PLANTS AND THE SULPHUR DISTRIBUTIONS OF THE PREPARATIONS

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BOTH from a general standpoint and from the particular one of nutrition, the compositions of the proteins which are present in the leaves of plants are of very considerable interest. In the present article, methods for determining sulphur-distributions, particularly in their bearing upon the estimations of cystine, cysteine and methionine, are examined and applied to several leaf protein preparations.

The leaf protein preparations numbered 1, 2, 3, 5, 6, 7, 8 and 10 in Table I were made during 1935 at Adelaide, South Australia, from fresh leaf material grown on plots which had not been manured for several months. The leaves were macerated directly [cf. Osborne & Wakeman, 1920; Chibnall & Schryver, 1921; Osborne *et al.* 1921; Chibnall, 1922] in a hand-driven corn mill at room temperature (about 20°) with the solvent intended to carry the protein. Ether-water was normally used as solvent [Chibnall & Schryver, 1921] as it was thought that plasmolysis by the ether might assist the dispersion of the protoplasm. The green juice was squeezed through a cloth, filtered through layers of filter paper (which did not retain the chloroplast material very well), and the protein was flocculated with acid at the apparent isoelectric point (about pH 4·5) and coagulated by heating the mixture to about 90° as described by Chibnall *et al.* [1933]. The coagulum was filtered off, washed with 0·0005 N HCl and, further to remove impurities, extracted successively with boiling alcohol, boiling 0·005 M citric acid (which usually reduces the ultimate ash content but removes only a negligible amount of nitrogenous substance), twice with boiling alcohol (or until extract was almost free from chlorophyll) and finally with warm ether.

In cases 3 and 6, some of the leaf material was ground with 10 % aqueous NaCl saturated with ether, instead of with ether-water as were the corresponding preparations, 2 and 5 respectively. Prep. 8 was made by extracting, but not grinding, with this solvent the leaf residues after extraction of 7. The object of using NaCl solution was to provide a satisfactory solvent for globulins in case they happened to be present. Microscopical examination of all the leaf residues revealed that large numbers of the cells had not been ruptured.

Prep. 9 had been made some years ago in this laboratory by the "used ether-water" method of Chibnall *et al.* [1933]. Prep. 4 was made by the same method from leaf material growing on a well-manured plot. The leaf material was first plasmolysed with ether-water which had already been used once for this purpose,

the readily diffusible constituents were removed by alternate imbibition of water and expression in a Büchner press, and the residue was then macerated with water in a motor-driven mincer. After squeezing the juice through cloth and filtering through a thick pad of paper pulp the protein was flocculated at about pH 4·5 and coagulated by heat. Impurities were removed from the coagulum by extracting with boiling water, alcohol, acetone and ether. The filtered leaf-juice was almost devoid of green colour, suggesting removal of most of the chloroplast material. Many of the leaf cells were not ruptured by the grinding.

The proteins were all denatured in the course of preparation. As regards the proteins properly associated with the plastids and the nuclei, it is improbable that they are adequately represented in preparations obtained by either of these procedures.

In Table I are shown, in the order of the columns: the number distinguishing the preparation: the plant species and date of extraction: the % dry wt. of the leaf material: the % total N (t), "albuminoid" N (a) and coagulable N (c) in the

Table I

No.	Source and date		% dry wt.	% N in dry leaf (t, a, c)	Extraction: Protein-N as % of leaf-N (t, a, c)	% N protein	% ash protein
1	*Dactylis glomerata* (cockfoot grass)	3. vi. 35	17·9	2·98 (t), 2·80 (a)	24·0 (t), 25·5 (a)	13·1	3·4
2	,,	7. xi. 35	19·0	3·20 (t), 2·82 (a)	24·4 (t), 27·7 (a)	13·0	1·0
3	,,	7. xi. 35	19·0	3·20 (t), 2·82 (a)	17·5 (t), 19·9 (a)	13·1	1·0
4	,,	29. ix. 36	12·5	6·81 (t), ·—	26·0 (t), --	14·1	1·1
5	*Phalaris tuberosa* (Toowoomba canary-grass)	20. viii. 35	20·4	3·08 (t), 2·72 (a)	13·4 (t), 15·2 (a)	15·4	0·7
6	,,	20. viii. 35	20·4	3·08 (t), 2·72 (a)	8·1 (t), 9·2 (a)	15·35	0·8
7	*Medicago sativa* (lucerne)	18. vii. 35	20·1	5·1 (t), 4·4 (a)	27·2 (t), 31·6 (a)	13·85	1·4
8	,,	18. vii. 35	20·1	5·1 (t), 4·4 (a)	0·95 (t), 1·1 (a)	13·85	1·7
9	,,	29. vi. 33	21·0	3·25 (t), 2·8 (c)	10·9 (t), 12·6 (c)	14·2	1·2
10	*Atriplex nummularium* (Oldman salt-bush)	6. xii. 35	15·6	3·48 (t), 2·54 (a)	13·1 (t), 17·9 (a)	12·1	4·5

dry-leaf material: the extracted protein-N as a percentage of the leaf-N (t, a, c): the % N in the protein preparation and finally the % of ash in the protein by ignition in air at about 600°. Materials dried in air at 85° were normally used in the analyses. Complete drying (for dry wt.) was done by heating at 105° followed by vacuum desiccation over conc. H_2SO_4. N estimations were made by the Kjeldahl method. The coagulable N was that which was not extracted from the finely-ground dried material at about pH 4·5 by two 10 min. extractions each with 100 times the weight of dilute hydrochloric or acetic acid at 100°, and the albuminoid N was that remaining after extraction under the Stutzer conditions ($Cu(OH)_2$ reagent).

Methods of determining the sulphur distribution

Assuming provisionally that the only S-containing amino-acids in the protein are cystine (and/or cysteine) and methionine, the distribution may be written: cystine-S + cysteine-S + methionine-S + other S = total S, and if "other S" is present entirely as SO_4 (as inorganic contaminant or attached to $-NH_2$ groups) and ethereal SO_4 (e.g. of prosthetic groups, if any), it should appear as inorganic SO_4 on prolonged acid hydrolysis at high temperature. In the absence of systematic errors it should thus be possible to check the provisional assumptions to within the limits imposed by the magnitudes of the random errors. The systematic errors, however, may be very large and they are, as yet, so uncertain

in some respects as to make a proper check of the above assumptions impossible. The sources of these errors have been classified roughly elsewhere [Lugg, 1933, 2; 1938] chiefly in relation to specific procedures such as hydrolysis, but it must be realized that sources associated with an assumed extensibility of knowledge of the behaviour of, say, a free amino-acid to the case of the corresponding amino-acid residue in the protein molecule, have an existence independent of procedure. In the following discussions they will be considered in their particular relationships to the estimations concerned.

The most specific methods of estimating cystine and cysteine present in solution appear to be the colorimetric ones. Employed in conjunction with acid (HCl, H_2SO_4) hydrolysis of "pure" proteins they have yielded valuable data, but it is known that errors may be associated with the hydrolysis of impure proteins. Bailey [1937] has shown that the losses resulting from HCl hydrolysis of edestin with arabinose are enormously greater than would be anticipated from hydrolysis tests of free cystine with arabinose and other carbohydrates [Lugg, 1933, 2], but the causes remain obscure. They may be connected with the formation, even temporarily, of sulphydryl groups by hydrolytic cleavage of disulphide bonds in edestin [Mirsky & Anson, 1934–5; Astbury *et al.* 1935; Mirsky, 1935–6], for in the presence of carbohydrates and their decomposition products the resulting mercaptal and mercaptole formation could effect large losses [Lugg, 1933, 2]. The procedure might be expected to lead to serious underestimation with the impure leaf proteins which normally contain substances of carbohydrate nature. It was found that the losses of cystine could not be averted by hydrolysing with the HCl-H.COOH reagent of Miller & du Vigneaud [1937] in place of HCl. Hydrolysis with alkali-stannite reagents could not be exploited, for whilst free cystine is almost quantitatively reduced to cysteine, edestin yields little more than half the cysteine to correspond with the cystine content of HCl hydrolysates.

Blumenthal & Clarke [1935], in a valuable contribution, estimated the following S-fractions in some protein preparations: sulphate-S after acid hydrolysis, labile S (i.e. appearing as PbS after reduction and digestion with alkali-plumbite), SO_4-S after oxidation with bromine-water, SO_4-S after oxidation with HNO_3 and non-SO_4-S after HNO_3 oxidation (i.e. by difference between total S and the preceding value). They tested the procedures, which do not permit discrimination between cystine and cysteine, with mixtures of cystine and methionine, and adjusted conditions so that the labile S and SO_4-S due to HNO_3 oxidation would correspond with the cystine present, and the non-SO_4-S after HNO_3 oxidation with the methionine, for whilst cystine-S is readily oxidized to SO_4 by HNO_3 methionine-S is only slowly oxidized beyond sulphoxide, sulphone or possibly methanesulphonic acid. Neither cystine nor methionine yield much SO_4 when treated with bromine water. As the estimated S-fractions in the proteins were not in complete conformity with the cystine and methionine contents estimated by fairly specific methods and did not show the same interrelationships as mixtures of cystine and methionine would have shown, and were often unaffected by working with HCl hydrolysates of the proteins instead of the proteins themselves, they attributed the discrepancies to the presence of small quantities of other S-containing amino-acids apart from cysteine. Such amino-acids may indeed occur in proteins, but the claim is based upon the assumption that the amino-acid residues behaved in the various procedures precisely as do the free amino-acids, and there is therefore the distinct possibility that unavoidable deficiencies of method suggested the presence of substances that did not exist in the proteins.

SO_4-S after HCl hydrolysis, SO_4-S after HNO_3 oxidation and total S estimations, which may be regarded as comprising "differential oxidation" data, were all made with the leaf proteins. The other S-fractions studied by Blumenthal & Clarke could not have been estimated satisfactorily in such preparations.

A further contribution to the study of the S-distribution in proteins was made by Baernstein [1936, 1, 2] who had earlier [1932; 1934] exploited Barger & Coyne's [1928] adaptation of the Zeisel procedure for the estimation of methionine. Baernstein found that in the course of digestion with conc. HI cystine was mainly reduced to cysteine and methionine was largely converted into methyl iodide and the thiolactone of homocysteine. Having established that the thiolactone ring would open and reduce tetrathionate to thiosulphate at mild alkalinity, he was able to devise iodine titrations for the thiolactone as well as for the cysteine. The scheme does not permit any discrimination between cystine and cysteine. He found also that SO_4 was reduced to H_2S and SO_2 by digestion with the HI. On this basis he claimed that almost all the S in a variety of proteins occurred in cystine, cysteine, methionine and SO_4, a claim since disputed by Bailey [1937]. From tests with mixtures of cystine, methionine, other amino-acids and small amounts of glucose, Baernstein believed that the procedure would be applicable without error to impure proteins.

It was found that Baernstein's [1936, 1] methods required modification before they could be applied at all satisfactorily to the impure leaf proteins, particularly in regard to the digestion with HI. His earlier (volatile iodide) method for the estimation of methionine cannot be considered in any way reliable with preparations containing polysaccharide impurity of unknown composition.

Details of procedures and tests

Hydrolysis with HCl. 2 g. samples of preps. 1 and 10 were heated with 15 ml. of $5N$ HCl at 100° for 24 hr. The mixtures were adjusted to pH 2·5 with NaOH, the insoluble humin was removed and the hydrolysates were adjusted to volume. The $RSSR$ [Lugg, 1932] and cystine [Lugg, 1933, 1] contents of the hydrolysates were estimated. The $RSSR$ estimations were performed without the aid of a compensating colorimeter to correct for the pronounced adventitious colorations, simply by making enough trials with the $HgCl_2$ and ferrous salt and then comparing the appropriate "ferrous salt standard" with a cystine standard colour solution. A compensating colorimeter was employed in the single cystine estimation. No RSH could be detected with certainty in the hydrolysates, but then no steps had been taken to prevent oxidation to $RSSR$, and in any case the impurities would have left very little RSH free.

Digestion with HI. Baernstein's [1936, 1] digestion procedure is to heat for 8 hr. at the boiling point in a current of nitrogen about 0·5 g. of the protein with 10 ml. of fresh, conc. HI (57 % HI, sp. gr. 1·7) from which the slight reddish coloration has first been removed by warming with 0·1 g. of KH_2PO_2. The procedure was tested with mixtures of cystine and methionine, the cystine titrations being about 2 % and the methionine about 10 % short of stoichiometrical requirements. Similar findings have recently been reported by Kassell & Brand [1938]. With recrystallized edestin the cystine plus cysteine and methionine contents, corrected for the above losses, checked fairly well with the $RSSR$ and methionine contents obtained by Bailey [1937] for a different sample of edestin. In the presence of 20 % by weight of arabinose, however, the coloration due to iodine was not permanently discharged and there were losses of about 9 % of the cystine plus cysteine and about 8 % of the methionine; these losses were

increased to about 13 and 19 % respectively (neglecting any effects of modifica-
tion in the absence of arabinose) by increasing the amount of KH_2PO_2 employed
from 0·1 to 0·5 g., which was more than sufficient to prevent the appearance of
iodine. Complete elimination of hypophosphite during the 8 hr. digestion period
proved to be entirely unsatisfactory, as also was the substitution of red phos-
phorus for KH_2PO_2.

The most satisfactory variation was to reduce the coloration of the HI to a
deep straw-yellow by heating with the minimum amount of KH_2PO_2 necessary,
and to commence the digestion of the protein with the addition of 0·02 g. of
KH_2PO_2, adding like amounts as digestion proceeded and with intervals of not
less than 2 min. between additions, so long as the iodine coloration continued to
develop. As a rule the intervals are found to be of several hours' duration after
the first few additions. It is desirable to avoid an excess of more than 0·02 g. of
KH_2PO_2 at any time, and the coloration due to iodine should never be much
more than noticeable. With impure leaf proteins the digest can be dark for
reasons other than the presence of iodine and care must be exercised in adding
KH_2PO_2. The volume of digest is most satisfactorily reduced later to about
3 ml. by evaporation under reduced pressure in a current of nitrogen with a
final 0·02 g. of KH_2PO_2, and if, on subsequent dilution to 25 ml., the solution is
not clear, it should be filtered in a nitrogen atmosphere. A small amount of
tarry "humin" develops during the digestion of impure leaf proteins and of
arabinose or pectin (especially if protein is present), and an orange coloured
flocculum appears when the evaporated digest is diluted. Incidentally, more
satisfactory end-points with starch as indicator are obtained in the titration of
the cysteine, by adding a slight excess of the iodate solution and instead of back-
titrating with the thiosulphate solution, adding a slight excess thereof and back-
titrating with the iodate.

Under these modifications mixtures of cystine and methionine showed
deficiencies to titration of about 2 % of the cystine and about 5 % of the
methionine, as judged by stoichiometrical requirements. Superimposed upon
the loss of 2 % of the cystine by titration, some 4 or 5 % of the reduced cystine
(cysteine) was modified in such a way (deaminated, decarboxylated?) that it
would not react with the highly specific Sullivan reagent. Baernstein [1936, 1]
states that the reaction, as used by Sullivan & Hess [1930], may be applied to the
cysteine in the diluted HI digests though not very satisfactorily. The reaction
was found to be affected by the presence of iodides and the appropriate substances
must therefore be introduced in preparing the standard used for colorimetric
comparison. Plain $HI-KH_2PO_2$ digests and $HI-KH_2PO_2$ digests of gelatin plus
tyrosine were used for this purpose, in conjunction with Lugg's [1933, 1] method[1]
based upon the Sullivan reaction, and due allowance was made for the minute
cystine content of the gelatin. The digests of gelatin plus tyrosine were used
when estimating the cysteine contents of protein digests, but were not entirely
satisfactory, colour-matching being imperfect.

The effects of the presence of 20 % of arabinose upon the estimations of
cystine plus cysteine and methionine in edestin were only slightly reduced by
introducing the new digestion procedure (see Table II). The effects of 20 % by
weight of "Collier" pectin were about half as great as those of arabinose. There
were substantial increases in the values obtained with the impure leaf proteins,
however. Blanks with arabinose and pectin were virtually zero.

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[1] Strangely enough it has not been recognized by some [e.g. Sullivan & Hess, 1936] that in this
method equimolecular amounts of cystine and cysteine yield substantially the same coloration, in
contrast to the erratic relationships obtaining in the Sullivan [1926] and Sullivan [1929] methods.

Differential oxidation

SO₄-S after HCl hydrolysis (i.e. without oxidation). 0·5 g. of the preparation was heated at 100° with 5 ml. of $5N$ HCl for 20 hr., and the mixture was adjusted to pH 2·5 with NaOH. After filtering, the filtrate and washings (with 0·01 N HCl) were treated with 0·3 ml. of $10N$ HCl and evaporated to 30 ml. on the water bath. 1·0 ml. of 0·5 M $BaCl_2$ solution was added and the solution was kept hot for 1 hr. and then allowed to cool and to stand for 12–15 hr. The $BaSO_4$ was collected by filtration and washed free from contaminants as judged by a negative chloride test with $AgNO_3$. It was ignited and weighed in the usual way.

SO₄-S after HNO_3 oxidation. 0·3 g. of the preparation was boiled with 8 ml. of "fuming" HNO_3 (sp. gr. 1·5, 95% HNO_3) for 36 hr. in a 100 ml. round-bottomed flask fitted with a "finger" condenser, and containing a small glass bead to promote even ebullition. The mixture was evaporated with 50 mg. of KNO_3 to dryness on the water bath, and twice re-evaporated to dryness after the addition of 2 ml. of $5N$ HCl each time. It was then taken up in water containing 0·3 ml. of $10N$ HCl and, after filtering, the filtrate and washings were evaporated to 30 ml. and $BaSO_4$ was precipitated and weighed as before.

Total-S (SO_4-S after peroxide-fusion). 0·2 g. of the preparation was treated with 5 ml. of 1·5 N NaOH in a nickel crucible and the mixture was evaporated to dryness at 105°, the drying surface being pricked occasionally to assist evaporation. It was then fused gradually under a mixture of 2 g. each of Na_2O_2 and Na_2CO_3 to a dull red heat, and a further 0·1 g. of Na_2O_2 was added before cooling. The melt was dissolved in warm water, treated with 0·1 g. of Na_2O_2 and acidified slightly by adding $10N$ HCl. After warming to expel free chlorine NaOH was added in slight excess (indicated by the persistence of precipitated nickel hydroxide) and then $10N$ HCl in 0·5 ml. excess over that required to dissolve the nickel hydroxide. The solution was filtered, filtrate and washings were evaporated to 50 ml. and $BaSO_4$ was precipitated and weighed as before.

All operations were made to conform as far as possible with standard plans, and blanks were determined by putting varying known quantities of Na_2SO_4 through the standardized operations (simple "reagent blanks" can be most misleading).[1] A few checks of the data quoted by Blumenthal & Clarke [1935] for the oxidation of cystine and methionine by HNO_3 proved satisfactory. Addition of 20% by weight of arabinose to edestin was without effect upon the values obtained with the protein itself. The presence of finely divided silica in HNO_3 digests of the impure leaf proteins may have been responsible for their occasional tendency not to boil smoothly.

RESULTS AND DISCUSSION

The protein portions proper of preparations which contain non-nitrogenous impurity are most readily compared in amino-acid composition by referring the analyses to a nitrogen basis. It is believed that none of the preparations contained more than a trace of nitrogenous impurity. The work was done with air-dried materials containing several % of moisture as a rule, but drying at 105° for 18 hr., contrary to Baernstein's [1936, 1] findings, did not affect the estimations detectably.

[1] Iron and aluminium, if present in appreciable quantity, must be removed from solution by the usual methods before precipitating the $BaSO_4$.

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The results of analyses of the leaf protein preparations are shown in Table II, and are prefaced by those of edestin (A) and edestin plus 20% by weight of arabinose (B). Some of the values are from single, others from duplicate and triplicate estimations. The random errors, as % of the values reported,

Table II

| No. | % N | HCl hydrolysis | | HI digestion | | | Differential oxidation | | | |
		% cyst.-N (RSSR)	% cyst.-N (sp.)	% cyst.-N (sp.)	% cyst.-N (titr.)	% meth.-N (titr.)	% cyst.-N	% meth.-N	% cyst.-N + % meth.-N	SO$_4$-S × 100/N
A	18·5	0·88*	0·82*	0·87	0·90	1·18	0·94	1·24	2·18	0·07
B	(15·4)	0·59†	0·59†	—	0·84	·1·09	0·94	1·24	2·18	0·07
1	13·1	0·14	—	—	1·38	1·21	1·51	1·44	2·95	0·33
2	13·0	—	—	—	1·28	1·20	1·47	1·35	2·82	0·27
3	13·1	—	—	—	1·28	1·19	1·53	1·47	3·00	0·19
4	14·1	—	—	1·4	1·50	1·30	1·68	1·49	3·17	0·40
5	15·4	—	—	1·5	1·50	1·45	1·63	1·48	3·11	0·52
6	15·35	—	—	1·4	1·40	1·36	1·58	1·44	3·02	0·89
7	13·85	—	—	1·1	1·11	1·22	1·19	1·23	2·42	0·29
8	13·85	—	—	—	-	—	1·31	1·23	2·54	0·34
9	14·2	—	—	—	1·21	1·31	1·29	1·35	2·64	0·41
10	12·1	0·74	0·52	1·15	1·30	1·55	1·44	1·53	2·97	1·92

Note. Quantities marked * are recalculated, with corrections for losses of the free amino-acids, from Bailey's [1937] data for a different sample of edestin containing 18·4% N but prepared from the same batch of hemp seed by the same method, and those marked † for edestin to which 10% by weight of arabinose had been added. Bailey found 20% loss of methionine after hydrolysing the edestin-arabinose mixture with HCl and removing the humin, and no loss in absence of arabinose, the % methionine-N by Baernstein's [1934] "volatile iodide" procedure being 1·23 (recalculated with correction). Baernstein [1936, 2] reports values by titration of HI digests for two samples of edestin, the values for one of the samples agreeing fairly well with those shown above in the table.

are believed not to exceed those indicated below in the description of the contents of the table. In the order of the columns are shown: the number or letter distinguishing the preparation (see Table I): the % N in it (error 0·5%, affecting all the other errors); and then a series of estimated cystine plus cysteine and methionine contents expressed as % of the total N appearing as these amino-acids and a few quantities incidental thereto. The series is: cystine plus cysteine from RSSR content (error 2% for A, 5% for the others), and by specific colori-metric estimation (error 3% for A, 5% for the others) of HCl hydrolysates; cystine plus cysteine by specific colorimetric estimation (error 4 or 5% for A, 8% for the others), cystine plus cysteine from titration (error 1 or 2% for A and B, 3% for the others), and methionine from titration (error 2% for A and B, 4% for the others) of HI digests; cystine plus cysteine (error 2% for A and B, 4% for the others) and methionine (error 3% for A and B, 5% for the others) from differential oxidation on the assumption that these are the only S-containing amino-acids and that their S appropriately appears as SO$_4$ in the successive stages of oxidation; the sum of the last two values (error 2%); and finally the SO$_4$-S as % of the weight of N in the material taken (error from 2% for the larger to 20% for the smaller values). All values have been corrected only for the losses to which the free amino-acids are subject in the procedures involved, and even these corrections, though small, are not rigorously applicable.

In the first place it will be noticed that the various procedures all lead to substantially the same values in the case of edestin (A), and in the second place that the values obtained with the impure leaf proteins vary with procedure in a manner reminiscent of the effects of adding arabinose to edestin (B). The

extremely low $RSSR$ content of the HCl hydrolysate of 1 recalls similar low cystine contents of cocksfoot leaf protein HCl hydrolysates reported by Pollard & Chibnall [1934]. From various considerations (extensive amino-acid analyses, the amounts of humin produced on acid hydrolysis etc.) it seems that the cocksfoot preparations, and probably those from other species of leaf too, should contain rather more than 16·5 % N if they were "pure", as against an earlier computation by Miller [1936] of 16·0 %, and that the usual organic impurity may be a pentosan, a pectin or a mucilage with the evidence mostly in favour of the last. There can be little doubt therefore that the major variations of the values with procedure, as shown in Table II, are due to the presence of organic impurities.

A greater amount of impurity may be expected to have a greater effect upon an estimation, and the degrees of vitiation may well depend upon the precise nature of the protein as well as that of the impurity. In the case of edestin itself (A), however, there are definite, small variations with procedure, and these may be attributed either to the presence of very small amounts of other S-containing amino-acids or to the deficiencies of methods of estimation which still involve the unwarrantable assumption that an amino-acid residue in protein behaves precisely as does the corresponding free amino-acid. Moreover, although the disparities between the values obtained by HI digestion and differential oxidation of the impure leaf proteins are generally greater than they are with edestin plus arabinose, it is justifiable to assume that the impurities would cause some loss in the former procedure. The differential oxidation procedure therefore, though less specific, may yield the more appropriate values both with edestin and with the impure leaf proteins. Complete acceptance of these values would imply an acceptance of the provisional assumptions as to the nature of the S fractions, recognition that the sum of the fractions thus estimated necessarily equals the estimated total S and a denial of the existence of systematic errors. At all events it seems extremely unlikely that the real cystine plus cysteine contents could have been appreciably lower than those obtained by HI digestion, because titration and colorimetric estimations check well.

Any doubt concerning the specificity of the differential oxidation procedure notwithstanding, the values obtained by it are of use in deciding whether one protein differs from another in amino-acid composition. The individual cystine plus cysteine and methionine values lack the precision which their sums possess, but these too may be used for the purpose, and it can be stated that the preparations made from one species of leaf were not all identical in composition, even when prepared from the same batch of leaf material.

The three preparations from lucerne leaves were of slightly lower cystine plus cysteine and methionine contents than the other leaf protein preparations were, but by the usual standards of animal nutritional requirements none could be adjudged deficient in its contents of these amino-acids.

SUMMARY

The preparation of some samples of protein from the fresh leaves of cocksfoot grass, Toowoomba canary-grass, lucerne and Oldman salt-bush, is described.

Methods of determining the S-distributions of the samples are discussed and described and the results of analyses are recorded.

It is pointed out that as the cystine (and/or cysteine) contents appear to lie within the range 1·1–1·7 %, and the methionine within the range 1·2–1·6 % of the protein-N, none of the preparations can be regarded as deficient in these amino-acids by the usual standards of the nutritional requirements of animals.

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CCLXXIII. THE AMIDE, TYROSINE AND TRYPTOPHAN CONTENTS AND THE SULPHUR DISTRIBUTIONS (CYSTINE PLUS CYSTEINE AND METHIONINE CONTENTS), OF SOME PLANT LEAF PROTEIN PREPARATIONS

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IT is the purpose of this article to record partial amino-acid and amide analyses of protein preparations made here and elsewhere from time to time from the fresh leaves of various plants at different stages of growth and growing under different climatic and manurial conditions, and to compare the preparations with one another. Some work done with a few other protein preparations is included from general interest.

The preparations

The leaf protein preparations are listed in Table 1, which shows in the order of the columns: the characteristic number or letter; the species of leaf material and date of extraction; the % dry wt. of the leaf material; the % total N (t), "albuminoid" N (a), coagulable N (c) and protein-N (p) in the dry leaf material; abbreviations indicating the method of extraction: the degree of extraction, usually represented by the extracted protein-N as % of the leaf-N (t, a, c or p, preferably the last, if known), but in some cases where these data are missing, by the weight of extracted dry protein as % of the leaf dry wt. (wt); the % N in the protein preparation; and finally, the % of ash in it. The methods of estimating most of the quantities referred to above have been described briefly in an earlier publication [Lugg, 1938, 2]. The "protein"-N in the leaf material was that left in the residue containing the coagulable N after further extraction with alcohol, dilute citric acid, alcohol and ether, as described for the purification of most of the protein preparations mentioned in the earlier article.

Of the abbreviations, "d" means direct maceration of the leaves [see Lugg, 1938, 2], and "$w1$" and "$w2$" mean maceration after washing the readily diffusible solutes from the leaf cells, "1" signifying that ether, and "2" that "used" ether-water, was used to plasmolyse the cells [Chibnall, 1923; Chibnall *et al.*, 1933]. Following these abbreviations, "w" means that water, "ew" that ether-water, "es" that ether-saturated NaCl solution, or "pH 7 (or 9, 10 or 11)" that a dilute buffer solution of the indicated pH value,[2] was the protein solvent with which the leaves were macerated; finally "f" and "c" respectively signify

[2] The pH values of the juices obtained were not estimated but were certainly lower than those of the buffers employed, juices obtained with water as solvent being of about pH 6. It is considered unlikely that the proteins were irreversibly dissociated.

Table I

Prep.	Source and date	% dry wt.	% N in dry leaf (t, a, c, p)	Method of extr.	Extent of extr.	% N prep.	% ash prep.
A	(Edestin)	—	—	—	—	18·5	0·2
B	(Casein)	—	—	—	—	15·05	2·0
C	(*Brassica campestris*) (Swede turnip)	—	—	—	—	13·0	16·4
1	*Dactylis glomerata* (cockfoot grass)	3. vi. 35	17·9	2·98 (t), 2·80 (a)	d, ew, f	25·5 (a)	13·1	3·4
2	,,	7. xi. 35	19·0	3·20 (t), 2·82 (a)	d, ew, f	27·7 (a)	13·0	1·0
3	,,	7. xi. 35	19·0	3·20 (t), 2·82 (a)	d, es, f	19·9 (a)	13·1	1·0
4	.,	29. ix. 36	12·5	6·81 (t), 4·50 (p)	w2, w, f	39·4 (p)	14·1	1·1
5	,,	27. v. 37	12·4	4·15 (t), 3·41 (p)	d, w, f	31·1 (p)	11·8	4·8
6	,,	27. v. 37	12·4	4·15 (t), 3·41 (p)	d, pH 7, f	37·6 (p)	12·05	3·6
7	,,	27. v. 37	12·4	4·15 (t), 3·41 (p)	d, pH 9, f	37·3 (p)	12·7	2·7
8	,,	27. v. 37	12·4	4·15 (t), 3·41 (p)	d, pH 11, f	41·2 (p)	12·7	2·2
9	,,	20. ix. 37	16·6	5·57 (t), 4·33 (p)	d, w, c	30·3 (p)	13·1	1·0
10	,.	20. ix. 37	16·6	5·57 (t), 4·33 (p)	, pH 10, c	5·7 (p)	13·2	4·8
11	,,	20. ix. 37	16·6	5·57 (t), 4·33 (p)	d, pH 10, c	36·7 (p)	13·1	1·2
12	*Phalaris tuberosa* (Toowoomba canary-grass)	20. viii. 35	20·4	3·08 (t), 2·72 (a)	d, ew, f	15·2 (a)	15·4	0·7
13	,,	20. viii. 35	20·4	3·08 (t), 2·72 (a)	d, es, f	9·2 (a)	15·35	0·8
14	*Lolium perenne* (perennial rye-grass)	6. xi. 33	18·1	4·6 (t), 3·8 (c)	w2, w, f	21·8 (c)	12·85	1·1
15	*Lolium italicum* (Italian rye-grass)	12. vi. 33	16·0	- ...	w2, w, f	4·8 (wt)	14·2	1·1
16	,,	26. vi. 33	15·0	5·9 (t), 5·2 (c)	w2, w, f	20·5 (c)	13·5	1·1
17	*Poa trivialis* (rough-stalked meadow-grass)	12. vi. 33	22·0	— ...	w2, w, f	2·3 (wt)	12·7	1·5
18	,,	27. vi. 33	14·0	6·2 (t), 5·3 (c)	w2, w, f	13·7 (c)	14·0	0·8
19	*Festuca rubra* v. *fallax* (Chewing's fescue-grass)	26. ix. 32	-	-	w2, w, f	17·8 (c)	14·25	0·8
20	,,	9. vi. 33	26·0	—	w2, w, f	1·3 (wt)	14·4	2·0
21	,,	29. vi. 33	15·0	—	w2, w, f	0·9 (wt)	14·2	1·7
22	*Cynosurus cristatus* (crested dog's tail grass)	12. vi. 33	24·0	- ..	w2, w, f	6·6 (wt)	14·1	1·3
23	*Medicago sativa* (lucerne)	18. vii. 35	20·1	5·1 (t), 4·4 (a)	d, ew, f	31·6 (a)	13·85	1·4
24	,,	18. vii. 35	20·1	5·1 (t), 4·4 (a)	, es, f	1·1 (a)	13·85	1·7
25	,,	29. vi. 33	21·0	3·25 (t), 2·8 (c)	w2, w, f	12·6 (c)	14·2	1·2
26	*Trifolium repens* (wild white clover)	18. x. 33	13·6	5·09 (t), 4·3 (c)	w2, w, f	28·0 (c)	13·1	1·5
27	*Trifolium pratense* (red clover)	23. x. 33	23·7	2·71 (t). 2·4 (c)	w2, w, f	24·3 (c)	12·75	1·6
28	*Phaseolus vulgaris* (runner bean)	7. x. 36	—	—	w2, w, f	—	13·3	3·2
29	*Atriplex nummularium* (old-man salt-bush)	6. vi. 35	15·6	3·48 (t), 2·54 (a)	d, ew, f	17·9 (a)	12·1	4·5
30	*Spinacia oleracea* (spinach)	1923	—	—	l, , f	ca. 2 (p)	15·5	1·3
31	,,	,,	—	—	w1, w	—	11·7	14·8
32	,,	..	—	—	w1, w, f	ca. 20 (p)	15·1	2·1

that the leaf juice, after squeezing through cloth, was either filtered through filter paper, or centrifuged for 30 min. in a field of 500 × gravity, before flocculating the protein. Whereas most of the chloroplast and nuclear material was removed with the cell debris by filtration, much of the chloroplast material was not removed by centrifuging. In one instance, 30, protein was flocculated from the solution carrying the readily diffusible cell constituents; in another, 31, protein was flocculated from juice which had not been centrifuged or filtered through filter paper; in two other instances, 10 and 24, residues of leaf material from the extraction of 9 and 23 respectively, were extracted with other solvents but without further maceration.

The preparations were made from leaves ranging in age from very young to quite old, and in general, as regards a single species, a high % dry wt. connotes old age and relative toughness and a high % total N (ages being similar) connotes good manuring. The proteins were extracted at room temperature (10–25°). With materials of much the same degree of toughness the macerating machinery (a mincing machine or a corn mill) permitted much the same degree of maceration. Even with the tenderest leaves, however, many of the cells were not ruptured. Preps. 14–22 and 30–32 were drawn from supplies made by Prof. A. C. Chibnall and co-workers in the past. Preps. 1, 2, 3, 4, 12, 13, 23, 24, 25 and 29 have been described in an earlier article [Lugg, 1938, 2] wherein their characteristic numbers were 1–10 in succession.

Prep. A was the sample of edestin used in earlier work [Lugg, 1938, 1, 2]; B was a sample of casein used by Vickery & White [1933] and was kindly supplied by Dr H. B. Vickery; and C, a protein obtained from the juice of macerated turnips (see Williams [1917]) and believed to have a high S content, was kindly supplied by Mr F. W. Foreman.

Methods of analysis

Amide. The general scheme behind the procedure for estimating this quantity (acid hydrolysis of amide linkages and estimation of the liberated NH_3) is to insure virtually complete amide hydrolysis with minimum deamination of peptides and amino acids (notably arginine). The problem has been studied with special care by Shore *et al.* [1936], and whilst the most appropriate estimate appears to require many estimations with increasing periods of hydrolysis and an approximate elimination (by extrapolation) of the contribution of NH_3 by the deamination reactions, reasonably representative values can be obtained by a standard period hydrolysis. The procedure adopted was to heat 0·1 g. of the preparation with 5 ml. of $2N$ HCl at 100° for 3 hr. and to distill the NH_3 off at pH 10·0 (borate buffer), by boiling under reduced pressure at 45°, into standard acid, the excess of which was back-titrated with alkali.

The procedure was checked with edestin and edestin plus 20 % by weight of arabinose, the carbohydrate being entirely without effect and therefore, presumably, introducing no new systematic errors. In comparison with values obtained by hydrolysing the leaf protein preparations for the standard (3 hr.) period, those obtained in a 2 hr. hydrolysis period were generally a few % lower, and in a 5 hr. period a few % higher; and by hydrolysing with $5N$ HCl at the boiling point for 20 hr. they were generally increased by some 20 %.

Tyrosine and tryptophan. 0·2 g. of the preparation was hydrolysed with alkali stannite reagent as described by Lugg [1938, 1] and tyrosine and tryptophan were estimated in the hydrolysate by Lugg's [1937] methods. Diiodotyrosine could not be detected in a few preparations by comparing with values obtained by plain alkali hydrolysis, and was assumed to be absent in all cases. Most of the leaf protein preparations yielded small amounts of extraneous phenol during hydrolysis and the legume preparations 23, 24, 25, 26 and 28 yielded some extraneous indole as well. These substances presumably had their origin in the impurities which appear almost always to contaminate such preparations, and the legume preparations therefore must have contained some nitrogenous impurity. This non-protein-N (presumably very small in amount) has been ignored in all calculations. Whereas the phenol was readily removed by the ether extraction advocated, the indole was not adequately removed either by the ether or the toluene extraction. It slightly affected both the tint and the fading rate in the tryptophan colour reaction and so, to an unknown but

apparently small degree, vitiated the tryptophan estimations in these cases. Correction had to be made for adventitious coloration, which was more marked in the tryptophan than in the tyrosine estimations. The tyrosine and tryptophan contents of A have already been reported [Lugg, 1938, 1].

S-distribution (cystine plus cysteine and methionine). The S-distributions of A, 1, 2, 3, 4, 12, 13, 23, 24, 25 and 29 and the methods employed have been discussed in an earlier article [Lugg, 1938, 2]. Estimations were made with the other preparations by the HI digestion (titration) and differential oxidation procedures, there described.

RESULTS AND DISCUSSION

The results are recorded in Table II. The amide and amino-acid contents are all reported on a "nitrogen basis", namely, the N contained in the chemical species as % of the N in the protein preparation. This scheme facilitates comparison of the protein moieties of impure preparations if the nitrogenous impurities are negligibly small, and they are believed to have been so here. The

Table II

Prep.	% N	% amide N	% tyr.-N	% try.-N	% cyst.-N (d.o.)	% meth.-N (d.o.)	% cyst. + meth.-N (d.o.)	SO₄-S × 100/N (d.o.)	% cyst.-N (HI t.)	% meth.-N (HI t.)
A	18·5	9·63	1·80	1·09	0·94	1·24	2·18	0·07	0·90	1·18
B	15·05	—	3·14	1·17	—	—	—	—	0·18	1·77
C	13·0	—	—	—	1·36	1·65	3·01	0·35	1·24	1·62
1	13·1	5·05	2·37	1·83	1·51	1·44	2·95	0·33	1·38	1·21
2	13·0	5·11	2·32	1·82	1·47	1·35	2·82	0·27	1·28	1·20
3	13·1	5·06	2·36	1·86	1·53	1·47	3·00	0·19	1·28	1·19
4	14·1	5·26	2·34	1·82	1·68	1·49	3·17	0·40	1·50	1·30
5	11·8	5·05	2·39	1·81	—	—	—	—	—	—
6	12·05	5·35	2·40	1·86	—	—	·	—	—	—
7	12·7	5·16	2·38	1·87	—	—	—	—	—	—
8	12·7	5·38	2·38	1·86	—	—	—	—	—	—
9	13·1	4·94	2·38	1·89	—	—	—	—	1·52	1·24
10	13·2	4·48	2·09	1·65	—	—	—	—	—	—
11	13·1	5·09	2·29	1·81	—	—	—	—	1·43	1·31
12	15·4	4·85	2·48	1·86	1·63	1·48	3·11	0·52	1·50	1·45
13	15·35	5·13	2·34	1·58	1·58	1·44	3·02	0·89	1·40	1·36
14	12·85	5·02	2·26	1·74	1·65	1·60	3·25	0·37	1·26	1·16
15	14·2	4·72	2·34	1·80	1·58	1·57	3·15	0·33	1·31	1·43
16	13·5	4·70	2·33	1·74	1·70	1·69	3·39	0·58	1·42	1·50
17	12·7	5·28	2·33	1·74	1·65	1·63	3·28	0·36	1·34	1·33
18	14·0	5·13	2·31	1·80	1·52	1·66	3·18	0·33	1·36	1·50
19	14·25	4·64	2·34	1·87	—	—	—	—	1·41	1·36
20	14·4	5·12	2·36	1·88	1·54	1·58	3·12	0·79	1·31	1·41
21	14·2	4·95	2·38	1·93	1·66	1·62	3·28	0·25	1·37	1·46
22	14·1	4·73	2·36	1·96	1·65	1·55	3·20	0·26	1·50	1·41
23	13·85	5·29	2·70	<1·98	1·19	1·23	2·42	0·29	1·11	1·22
24	13·85	5·55	2·72	<1·88	1·31	1·23	2·54	0·34	—	—
25	14·2	5·16	2·74	<2·00	1·29	1·35	2·64	0·41	1·25	1·31
26	13·1	5·68	2·49	<1·88	1·23	1·33	2·56	0·30	0·99	1·23
27	12·75	5·40	2·50	1·70	1·25	1·29	2·54	0·27	1·19	1·22
28	13·3	5·08	2·36	<1·55	1·30	1·26	2·56	1·22	1·05	1·01
29	12·1	5·98	2·55	1·74	1·44	1·53	2·97	1·92	1·30	1·55
30	15·5	5·76	2·57	1·43	—	—	—	—	1·43	1·09
31	11·7	5·36	2·63	1·72	—	—	—	—	1·22	1·27
32	15·1	5·64	2·71	1·69	—	—	—	—	1·37	1·30

results have been corrected for the losses undergone by the pure, free amino-acids in the procedures concerned. The random errors of estimation are believed to lie within the limits indicated in the following description of the table's contents. In the order of the columns are shown: the characteristic number or letter;

the % N in the preparation (error 0·5 %, affecting all the others); the amide content (error 1 % for A, 1·5 % for the others); the tyrosine content (error 1 % for A and B, 2 % for the others); the tryptophan content (error 1·5 % for A and B, 3 % for the others, excepting what were almost certainly over-estimations at least several % in the case of 23, 24, 25, 26 and 28); the cystine plus cysteine content by differential oxidation (error 2 % for A, 4 % for the others); the methionine content by differential oxidation (error 3 % for A, 5 % for the others); the sum of these last two quantities (error 2 %); the SO_4-S as % of the weight of N in the preparation (error from 2 % for the larger to 20 % for the smaller values); the cystine plus cysteine content by titration of HI digests (error 1·5 % for A, 3 % for the others); and finally the methionine content by titration of HI digests (error 2 % for A and B, 4 % for the others).

The differences between the cystine plus cysteine and methionine contents estimated by titration of HI digests and by differential oxidation, have already been discussed in relation to some of the preparations [Lugg, 1938, 2]. The disparities are again apparent with the rest of the preparations and the same uncertainty as to their significance remains. Again it may be pointed out that while the estimations by titration of HI digests are much the more specific they are likely to be low, and that the values obtained by differential oxidation must be used when comparing compositions. SO_4-S values are regarded here simply as measures of some impurity.

Whereas significant differences in composition, even in respect of only one chemical species, prove the non-identity of the protein moieties of two preparations, their absence leaves only an indeterminate degree of probability (increasing with the number of chemical species considered) that they are identical.

Of the preparations from cocksfoot grass, 5, 6, 7 and 8 show that slightly increased extraction may result when the pH of the extracting liquid is increased beyond that of the mixed cell contents. 5 is of slightly lower amide content. Incidentally, when leaves of the Gramineae are macerated alone or with water the resulting juice is usually found to be of about pH 6. Prep. 10, made by extracting the leaf residue from 9 with a pH 10 buffer, is of materially lower amide, tyrosine and tryptophan contents than any of the other cocksfoot preparations which, except for small differences, are remarkably uniform in composition. In fact, except for 10, all the preparations from leaves of the Gramineae (1–22) show relatively small variations in composition, the greatest being in the S-distributions and the amide contents.

In comparison with these, the preparations from leaves of the Leguminosae (23–28) are rather less uniform in composition, but in general, their tyrosine contents are a little higher and their cystine plus cysteine and methionine contents are lower; and the preparations from leaves of the Chenopodiaceae (29–32) are generally of higher amide and tyrosine contents.

No definite variations in composition of extracted protein can be associated with the age of the leaves or with the climatic and manurial conditions during growth. It is to be noted too, that preps. 9, 11 and 30, which presumably contained more representative amounts of the proteins associated with the chloroplasts, do not differ markedly in composition from the others.

Taken as a group for comparison with preparations like A and B, the leaf protein preparations from monocotyledons (1–22) and dicotyledons (23–32) appear from these analyses to be of fairly uniform composition, and judged by the usual standards of animal nutritional requirements are by no means deficient in their contents of tyrosine, tryptophan, cystine plus cysteine and methionine.

The analyses of A and B are in reasonable agreement with the more recently published work of others on edestin and casein preparations. There appears to be nothing remarkable about the S distribution of C (from Swede turnips), but then, contrary to expectation, its S content was not abnormally high.

SUMMARY

Estimated amide, tyrosine, tryptophan, cystine (and/or cysteine) and methionine contents of protein preparations made from the fresh leaves of various mono- and di-cotyledonous plants, are recorded, together with those of a few other protein preparations.

The leaf protein preparations were made from *Dactylis glomerata*, *Phalaris tuberosa*, *Lolium perenne*, *L. italicum*, *Poa trivialis*, *Festuca rubra* var. *fallax* (Hack) and *Cynosurus cristatus*, of the Gramineae; *Medicago sativa*, *Trifolium repens*, *T. pratense* and *Phaseolus vulgaris*, of the Leguminosae; and *Atriplex nummularium* and *Spinacia oleracea*, of the Chenopodiaceae. For the most part they represented some 10–40 % of the leaf protein, but were probably rather deficient in the proteins associated with the plastids and nuclei of the leaf cells.

In comparison with preparations from the first plant order, those from the second and third were generally a little higher in tyrosine contents, those from the second a little lower in cystine (and/or cysteine) and methionine contents, and those from the third a little higher in amide contents. The ranges over all the leaf protein preparations were: 4·70–5·98 % amide-N, 2·09–2·74 % tyrosine-N, 1·43–1·98 % tryptophan-N, 0·99–1·70 % cystine (plus cysteine)-N, and 1·01–1·69 % methionine-N, the lower limits in the last two cases probably being underestimations, and the upper limits being possibly too high owing to lack of specificity in estimation in these same cases.

In regard to their contents of the amino-acids here estimated, the leaf protein preparations compare favourably with many other proteins which enter into the dietary of animals.

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CCLXXIV. ON CERTAIN SIMPLE PEPTIDES OCCURRING IN MARINE ALGAE

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SOME time ago Haas & Hill [1931; 1933, 1] described the isolation of water-soluble peptides from the brown alga *Pelvetia canaliculata* forma *libera* S. M. Baker and the red alga *Corallina officinalis* Linn. It was then suggested that the presence of peptides in algae was due to a lack of metabolic balance determined either by desiccation or by low illumination. To obtain further evidence in support of this it was decided to examine as many algae as possible and preferably calcareous forms, as these, owing to their incrustation, would naturally be exposed to a much reduced illumination. Accordingly the following were selected: *Corallina squamata* Ellis, *Lithophyllum incrustans* Foslie, *Amphiora capensis* Aresch and *Galaxaura subverticillata* Kjell. A number of unencrusted algae were also examined, but, so far, the only two found to contain peptides were *Pelvetia canaliculata* forma *libera* and *Griffithsia flosculosa*.

The peptides contained in the above calcareous algae and that of *Pelvetia* are described in the present communication with special reference to the number and nature of their constituent amino-acids, but an account of the observations made on *Griffithsia flosculosa* will be the subject of a future paper.

Methods

The general procedure for the isolation of the peptides was as follows. The air-dried material was digested three times in water over a boiling water bath for about 30 min. The resulting extract was poured off and the residual plant material freed from adherent liquid in a tincture press. The combined extracts were next precipitated with basic lead acetate, and the filtrate, after freeing from Pb with H_2SO_4, was treated with $Ba(OH)_2$ to remove SO_4 and was concentrated under reduced pressure; the solution was then precipitated by mercuric acetate, and the Hg salt, after washing, decomposed with H_2S, filtered from HgS and evaporated to dryness in a vacuum.

Nature and properties of the peptides

The peptides prepared as above are all amorphous, more or less viscous substances, varying in colour from a light lemon-yellow to a dark brown. They all give a green colour with Bial's reagent for pentose, but the intensity of the reaction varies, being least for *Pelvetia* and highest for *Lithophyllum*. Although detectable by means of the above colour reaction, the actual amount of carbohydrate was too small to estimate in the material available.

The peptides all give the biuret reaction, the colour varying from a reddish mauve to a blue-violet colour, but as the tint varies somewhat with dilution the reaction is of no value for characterization.

With the object of obtaining some information on the number of amino-acid molecules forming the peptide chain, the ratio of amino-N before and after

hydrolysis was determined. In order to eliminate the disturbing influence of NH_3 in the determination of amino-N, the following procedure was adopted.

A given amount of peptide was made up to a known volume, and two equal aliquots were measured out. One was then placed in a flask with dilute aqueous Na_2CO_3 and heated at 60° while a rapid current of air was drawn through. After all ammonia had been removed, the contents of the flask were carefully washed out, neutralized with acetic acid, evaporated and finally washed into a graduated flask and made up to 25 ml. The amount of amino-N in this solution was then determined by the Van Slyke method.

The other aliquot was heated with twice its volume of conc. HCl in an autoclave at 105° for 4 hr. After evaporating the HCl the residue was dissolved in water, made alkaline with Na_2CO_3 and heated over a water bath until no more NH_3 was evolved; it was then acidified with acetic acid, made up to 25 ml. and the amino-N determined.

From these two determinations the number of amino-acid groups in the peptide chain was obtained.

Products of hydrolysis of the peptides

The hydrolyses were carried out as described above and the dark brown solutions obtained were evaporated to dryness over a water bath to remove excess of acid, taken up with water and filtered from humic matter. As the quantity of material available was relatively small, the ordinary large scale methods for isolating and identifying the various acids were impracticable, and for this reason the tests employed are given in detail.

PELVETIA CANALICULATA forma LIBERA. This alga was collected at Blakeney Point, Norfolk, during the month of April.

The crude peptide obtained was a light lemon-yellow syrup; the yield, calculated on the air-dried alga, was 0·728 %.

After hydrolysis and removal of excess acid by evaporation the solution was tested for phenylalanine (Kapeller-Adler test) and tyrosine (Mörner's test), in both cases with negative result.

After removal of ammonia, a portion of the resulting solution was tested with phosphotungstic acid and, as no precipitate resulted, the remaining solution was concentrated to small bulk and saturated with HCl to remove glutamic acid, whose presence among the products of hydrolysis had already been established on an earlier occasion [1931].

In order to determine whether any other amino-acid remained after complete removal of the glutamic acid, the filtrate was concentrated, again saturated with HCl and once more filtered from a small quantity of precipitated glutamic acid hydrochloride; it was then boiled to drive off the acid, diluted and heated with excess of ZnO. After 40 hr. the solution was filtered and found still to give a strong ninhydrin reaction. A control experiment on authentic glutamic acid treated in the same way gave no ninhydrin reaction, showing that glutamic acid can be completely removed by this method. It was therefore concluded that a second amino-acid must be present. The solution was accordingly concentrated, freed from NH_3 and treated with a few crystals of phenol and an excess of NaOCl; an immediate deep blue colour resulted, suggesting glycine, alanine or leucine. To distinguish between these a little of the concentrated solution was treated with a few drops of saturated copper acetate; as no precipitate resulted, glycine and leucine were excluded, and it only remained to establish the presence of alanine. To this end the solution was deaminated with HNO_2 and tested for the presence of lactic acid, after treating with conc. H_2SO_4, by Hopkins' test with

copper sulphate and thiophene. A positive result was obtained, thereby establishing the presence of alanine.[1]

The ratio of amino-N before and after hydrolysis was found to be 11. In the earlier communication [1931] this ratio was given as 8 and the only acid detected was glutamic acid. Whether this discrepancy is due to seasonal variation or not is now under investigation.

The five following algae are all encrusted forms, but, as will be seen from the results described below, there is considerable variation in the composition of their constituent peptides.

CORALLINA SQUAMATA. The material used for extraction of the peptide was a composite sample, collected at different times of the year, from Dancing Ledge, Dorset.

The peptide was a dark amber-coloured substance of a resinous consistency, which however readily melted, on warming, to a viscous liquid. The yield of crude peptide calculated on the dry weight of the alga was 0·1 %.

After hydrolysis, the solution was treated with phosphotungstic acid; the heavy precipitate produced was washed and suspended in acetone-water and treated with excess of baryta solution: a rapid current of air was drawn through until all NH_3 had been removed; excess of baryta was removed with CO_2, and the resulting solution was shown to contain arginine by both the Sakaguchi test and Harden's diacetyl test.

The filtrate from the phosphotungstic acid precipitate was then treated with baryta to remove excess phosphotungstic acid and the precipitate, consisting of Ba phosphotungstate and $BaSO_4$, was filtered off. The filtrate gave only a feeble ninhydrin reaction, which suggested adsorption on the precipitate; the latter was therefore boiled with a strong solution of Na_2CO_3, and filtered; the filtrate now gave a strong positive reaction. After neutralization it was tested for aspartic and glutamic acids, but with negative results. On the other hand it gave a blue colour with phenol, followed by NaOCl, indicating glycine, leucine or alanine. Since it gave no precipitate with copper acetate the two former acids were excluded. The presence of alanine was, however, established by deamination with HNO_2, followed by Hopkins' thiophene test for lactic acid.

The test for phenylalanine (Kapeller-Adler) was negative.

The failure to find aspartic acid, after repeated trials, among the products of hydrolysis of this alga is significant in view of the fact that aspartic acid was definitely established in the other species, namely *Corallina officinalis*, as reported on an earlier occasion [Haas & Hill, 1933, 2].

The ratio of amino-N before and after hydrolysis of the peptide of *C. officinalis* was 4.

LYTHOPHYLLUM INCRUSTANS. The material used for this investigation was collected at Studland Bay, Dorset, in May, by chipping off the mauve superficial layer covering the chalk which is exposed only at low spring tide.

The thickness of the actual algal covering was only a few mm. and the main bulk of the sample was composed of the rock substratum. The material was crushed into small fragments and extracted three times with hot water, the aqueous extract being worked up in the usual manner. The weight of crude peptide obtained from different samples varied considerably from 0·055 to 0·185 %. These figures are only approximate and depend entirely upon the amount of chalk or limestone adhering to the weed.

[1] A separate experiment showed that authentic phenylalanine when treated as above likewise gives a positive reaction. Since however the solution obtained from the hydrolysed peptide failed to give the Kapeller-Adler reaction for phenylalanine it was concluded that the presence of alanine was established.

The crude peptide was a dark brown substance of a gummy consistency. After hydrolysis it gave a precipitate with phosphotungstic acid, which after the usual treatment yielded a residue which gave good positive tests for arginine by the Sakaguchi test and by Harden's test with diacetyl. The filtrate after the usual treatment for the removal of Ba gave a positive reaction for phenylalanine (Kapeller-Adler).

The ratio of amino-N before and after hydrolysis was 4.

AMPHIORA CAPENSIS. This alga is a South African species allied to *Corallina*.

The air-dried material, extracted in the usual manner, yielded just under 0·3 % of crude peptide, in the form of a dark brown semi-solid residue with a peculiarly unpleasant smell.

The amount of material available was, unfortunately, not sufficient to allow of a detailed examination, and the only amino-acid which could be definitely established was arginine. The filtrate remaining after removal of the arginine gave a blue colour with phenol and NaOCl, suggesting glycine, alanine or leucine. The addition of saturated copper acetate to the concentrated solution gave a turbidity on standing, much as leucine does, but it was not found possible to establish the presence of this amino-acid conclusively. Lack of material rendered it impossible definitely to exclude glycine or alanine, or to make a determination of the ratio of amino groups before and after hydrolysis.

GALAXAURA SUBVERTICILLATA. This is an encrusted green alga from Florida.

Extraction by the usual method gave a yield of about 0·2 % of a dark brown semi-solid peptide. The amount of material available was small, and only one amino-acid, namely phenylalanine, was definitely established. The hydrolysed product was precipitated with phosphotungstic acid without previous removal of ammonia; the resulting precipitate gave a negative test with ninhydrin, and as it gave no positive test for proline it was rejected.

The filtrate from the phosphotungstic acid precipitate was treated with baryta to remove excess of phosphotungstic acid and filtered. The filtrate was freed from Ba and precipitated with mercuric acetate. The resulting precipitate was decomposed with H_2S and filtered. This filtrate gave an amorphous precipitate with copper acetate, which was not further characterized owing to lack of material. The filtrate from the mercuric acetate precipitate, however, gave positive tests for phenylalanine. It may therefore be concluded that *Galaxaura* peptide contains at least two amino-acids of which one is phenylalanine.

The ratio of amino-N before and after hydrolysis was 2.

In addition, a further calcareous alga of South African origin, namely *Cheilosporium corymbosum*, was examined, but the amount of material available was too small for detailed examination. All that was established was that the weed yielded about 0·1 % of crude peptide in the form of a dark brown resinous material in which the ratio of amino groups before and after hydrolysis was 2.

For convenience of reference the results obtained are summarized in the following table:

	Alanine	Arginine	Aspartic acid	Glutamic acid	Phenyl-alanine
Amphiora capensis	+	+	.	.	.
Corallina squamata	+	+	.	.	.
Corallina officinalis	.	.	+	.	.
Galaxaura subverticillata	.	.	.	.	+
Lithophyllum incrustans	+	+	.	.	.
Pelvetia canaliculata forma *libera*	+	.	.	+	.

Although various other amino-acids were tested for, only those which gave positive results are recorded in the above table.

SUMMARY

1. The occurrence of peptides is reported in marine algae: *Pelvetia canaliculata* forma *libera* (Phaeophyceae), *Corallina officinalis*, *Corallina squamata*, *Amphiora capensis*, *Cheilosporum corymbosum*, the two latter from South Africa, *Lithophyllum incrustans* and *Galaxaura subverticillata* from Florida. Of these algae all except the first are encrusted forms.

2. The amount of crude peptide occurring in the algae varies from 0·05 to 0·29 % of the dry weight; in *Pelvetia*, however, the amount is as high as 0·728 %.

3. The peptides are all water-soluble and diffusible substances; they all contain a small proportion of pentose sugar.

4. The following amino-acids have been shown to occur in these compounds: alanine, arginine, aspartic acid, glutamic acid and phenylalanine.

5. In one genus, namely *Corallina*, a difference in composition of the peptide has been noted in different species, *C. officinalis* containing aspartic acid, while *C. squamata* does not, but contains instead alanine.

6. The number of peptide linkages as expressed by the ratio of amino groups before and after hydrolysis varies in the different peptides.

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CCLXXV. VITAMIN E DEFICIENCY IN THE RAT

III. FERTILITY IN THE FEMALE

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THE consumption of a diet free from vitamin E produces sterility both in the male and in the female rat. In the male the sperms become non-motile and this is followed by degeneration of the testes. The condition in the male is not reversible and cannot be cured by administration of vitamin E once the degenerative phase has been reached [Evans, 1925; Mason, 1926; Evans & Burr, 1927]. In the female it has generally been accepted that ovulation, fertilization and implantation remain normal, but pregnancy cannot be carried to term unless the animal is given an adequate dose of vitamin E on mating.

Bacharach *et al.* [1937] and Bacharach & Allchorne [1938] observed that the incidence of fertile matings among vitamin E-deficient rats was very much lower in those which had undergone a resorption-gestation than in virgin animals; they concluded that the process of resorption left some permanent damage to the reproductive system but made no histological examination. Martin & Moore [1936] reported the brown appearance of the uterus of rats fed on a vitamin E-deficient diet. Evans [1928] found that the incidence of spontaneous deciduomata of the uterus was much higher in vitamin E-deficient than in normal females.

In this laboratory it has been found that when vitamin E-deficient rats are mated with normal males and a vaginal plug is formed, this mating is almost invariably followed by implantation. I have found no apparent difference between virgin and resorbed animals in this respect. I do, however, find that among vitamin E-free animals, whether virgin or resorbed, more than half do not mate or else no plug is formed. It has also been found, in agreement with Bacharach, that animals which have once resorbed require a larger dose of vitamin E to prevent resorption in the next gestation than their litter-mate virgin sisters of the same age.

A post-mortem examination has been made of rats which have been used for vitamin E experimental work and it has been found, without exception, that every rat which has undergone a resorption-gestation has a brown or yellow-brown uterus and ovaries containing large corpora lutea. Histological examination of the uterus reveals occasional patches of fatty degeneration in both the longitudinal and circular muscle coats. Deposits of red-brown pigment are found throughout the muscle layers.[1] Many fibres are degenerated and packed with granules of pigment. These findings account for the discoloration of the uterus. There is often some fibrous tissue in the muscle layers, particularly in the older rats.

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[1] Since the completion of this paper Martin & Moore have described the finding of granules of pigment in the uterine muscle of their vitamin E-deficient rats (*Chem. & Ind.* 15 Oct. 1938).

A number of virgin animals which had been fed on the vitamin E-free diet for different periods of time have been killed and examined *post mortem*. From these examinations it has been found that similar changes take place in virgin, vitamin E-free rats, but that resorption greatly accelerates their onset. Table I

Table I

No. of rats	Months on vitamin E-free diet	History	Appearance of uterus	Microscopic examination of uterus
5	4	1 resorption	Pale yellow; 1 animal had small fibroid	Some pigment in muscle layers
4	4	Virgin	Thread-like, very pale yellow; 1 brownish-yellow and infected	Traces of pigment
5	5	1 resorption	Yellow-brown; 1 showed some thickening	Considerable amount of pigment
3	5	Virgin	Yellow-brown; 2 had fibroids and were bleeding	Considerable amount of pigment
7	7	1 or more resorptions	Yellow-brown to dark orange-brown, 1 infected	Large amount of pigment
6	7	Virgin	Yellow-brown	Less pigment than resorbed animals, no fibrosis
9	8	1 or more resorptions	Brown to dark brown; 1 bleeding from fibroid, 2 infected, 3 fibrosis; all enlarged	Large amount of pigment, extensive muscle degeneration
5	8	Virgin	Pale orange-brown; 1 very distended	Pigmented, a number of pigmented and degenerated muscle fibres present; some fat and in one case mucosa oedematous

illustrates these points; it is composed from the records of 44 consecutive post-mortem examinations. After 4 months on the diet there is a slight increase in the size of the uterus and definite, although slight, discoloration; after 5 months the discoloration and enlargement are more marked and the condition is severe after 6 months of vitamin E deficiency. The uterus of a rat which has been on the diet for 5 months and undergone a resorption-gestation shows more discoloration than that of a similar virgin animal. The amount of discoloration present was in each case comparable with the amount of pigment found on microscopic examination. The enlarged condition is not due to infection, although this may sometimes occur. Where there is no infection the enlargement is usually associated with fibrosis and thickening of the uterine wall. The infections which quite frequently occur are possibly due to the frequent vaginal examinations which are made, although every reasonable precaution is taken to avoid infecting the animal.

In view of the changes in the uterine muscle it is not surprising that vitamin E-deficient animals which have undergone a resorption-gestation require more vitamin E to enable them to produce a litter than similar animals which have not resorbed, and it can also be understood that if the uterine degeneration has proceeded far enough, pregnancy will be impossible. Table II illustrates these points. It will be seen that 0·35 ml. of a (diluted) vitamin E preparation was an inadequate dose for a virgin animal which had been on the vitamin E-free diet for 9 months, but 0·5 ml. produced a litter of 6 in a similar animal. 0·5 ml. was inadequate for animals of almost the same age which had undergone one or two resorptions respectively, yet 0·75 ml. produced a litter of 1 in an animal which

Table II

Rat no.	Dose of vitamin E preparation ml.	Result	No. months on vitamin E-free diet	No. resorption gestations	Appearance of uterus
1272	0·35	Litter of 5	5	1	Slightly yellow
1136	0·35	Litter of 2	8	0	Not killed
1096	0·35	Litter of 8 (dead in uterus)	9	0	8 full-term dead foetuses removed
1022	0·50	Litter of 6	9	0	Small and brown
794	0·50	Resorption	10	1	Brown; signs of resorption of large foetus
1033	0·50	Resorption	10	2	Large and very brown
698	0·50	Resorption	11	1	Large and very brown
1051	0·75	Litter of 1	10	2	Normal colour, very small
1032	0·75	Resorption	10	2	Dark orange-brown

had had two resorption-gestations and been on the diet for 10 months, but failed to prevent resorption in an exactly similar animal. Since the 0·35 ml. dose was adequate for the 3- and 4-months animals then 0·75 ml. is a relatively enormous dose and the fact that it failed to produce a litter in an animal which had received the vitamin E-deficient diet for 10 months and gave only a litter of one in another animal which had been on the diet for the same length of time, shows that after about 10 months of vitamin E deficiency female rats have reached or are very near the stage of irreversible sterility. On a normal diet a female rat is capable of producing living young until she is at least 18 months old. The normal colour of the uterus of the rat which had a litter of 1 following a dose of 0·75 ml. remains unexplained unless the large amount of the vitamin given exerted a curative effect on the degeneration. Investigation of this point is at present in progress.

The fact that fibrosis of the uterus often occurs in connexion with the discoloration has already been mentioned, but more advanced stages than this are regularly met with in this stock of vitamin E-deficient rats. Non-pregnant animals have occasionally been found bleeding from the vagina, the amount of blood lost being quite large in some cases. 30 cases of such bleeding have been observed in the last year and reference to Table I will give some idea of its incidence. These animals have been killed and the uterus has been found to have a series of rounded whitish enlargements separated by discoloured muscle. These masses are hard to cut and their inner surfaces are pearly white. On microscopic examination they are seen to consist of a mass of fibrous tissue and are typical fibromyomata.

It is, of course, quite possible that fibromyomata occur in rats fed on a normal diet, but the incidence among our stock must be very much less than among the vitamin E-deficient rats in this laboratory, because a post-mortem examination is made of all the stock animals which die or which are of no further use for breeding, and no tumour of this type has been found on microscopic examination. Similarly, these stock animals never develop uterine discoloration and even among those animals which are too old to bear litters no pigmentation or fatty degeneration of the uterus has been found. These observations are based on the records of our breeding stock for the last 2 years. This stock has been maintained at a level of approximately 150 females, 100 of which have been killed in the interval.

These observations raise several points. It is clearly important in assaying a substance for its vitamin E activity to make sure that the animals used have been

fed on the vitamin E-deficient diet long enough to be sterile but not long enough to have developed uterine changes, and the onset of these degenerative changes must obviously vary between different stocks and depend to a great extent on the degree to which the diet is free from vitamin E.

The statement that vitamin E deficiency is reversible in the female but not in the male is only partly true, because the deficiency is curable in both male and female rats if vitamin E is given early enough, but if the deficiency is allowed to proceed to testicular or advanced uterine degeneration, absolute sterility is produced in both sexes.

A more detailed study is being made of the incidence of the fibromyomata but this preliminary work indicates that in the rat they are associated with vitamin E deficiency.

SUMMARY

The discoloration of the uterus described by Martin & Moore in vitamin E-deficient rats has been confirmed.

This discoloration is caused by the deposition of a pigment in the muscle layers associated with degeneration of the muscle.

Prolonged vitamin E deficiency caused fibrosis of the uterine muscle: a certain number of the animals developed fibromyomata of the uterus.

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CCLXXVI. THE CHLORINE-SODIUM SULPHITE COLOUR REACTION OF WOODY TISSUES

II. THE BEARING OF THE COLOUR REACTION ON THE CONSTITUTION OF HARDWOOD LIGNIN

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IN a previous communication from this Laboratory [Campbell *et al.* 1937] it was shown that the well known colour reaction given by woody tissues with sodium sulphite following chlorination was not characteristic of lignin only but could also be given by certain tannins. It was concluded that the reaction was probably specific for phenolic compounds containing the 1:2:3-trihydroxybenzene nucleus but, although such a nucleus is known to occur in the gallotannins, there is no evidence that it occurs as such in native lignin. Great interest is therefore attached to the isolation by Freudenberg [1938] of substances containing a modified 1:2:3-trihydroxybenzene nucleus such as syringic acid and also to the identification by Leger & Hibbert [1938] of pyrogallol 1:3-dimethyl ether among the degradation products of lignin. It was considered that, if any of these substances could be shown to give the characteristic colour reaction with chlorine and sodium sulphite, additional proof would be forthcoming for the occurrence in native lignin of a modified trihydroxybenzene nucleus as a recurring unit for the reason that, during the delignification of hardwoods by the Cross and Bevan procedure, the coloration persists until the last traces of lignin have been removed. This would, of course, involve the assumption that lignin is composed for the most part of aromatic units. Until the publication of the results of Harris *et al.* [1938] such an assumption could not be maintained with any certainty. In their study of the products of the hydrogenation and hydrogenolysis of hardwood lignin these authors have confirmed and extended the work of Moldavskiǐ and Vaǐnshteǐn [1935] and proved beyond doubt that the greater part of lignin is aromatic in character. In the present study chlorine and sodium sulphite have been applied to a further range of substances, particular attention being paid to products obtained by Freudenberg [1938] in view of their importance as likely building units in lignin.

EXPERIMENTAL

The following substances were exposed to gaseous chlorine followed by sodium sulphite in the manner previously described [Campbell *et al.* 1937] with the results shown in Table I.

The faint reaction of pure 1:2:3-trimethoxybenzene is probably associated with the low solubility of this compound in water and the consequent difficulty in securing its optimal reaction with chlorine. It was observed that of all the substances so far tested gallic, trimethylgallic and syringic acids gave colorations

Table I. *Colour reactions of various phenols and their derivatives
with chlorine and sodium sulphite*

Substance	Colour reaction
Guaiacol	Nil
Vanillin	Reddish brown
Veratrole	Nil
Eugenol	Very faintly positive
Gallic acid	Strongly positive
1:2:3-Trimethoxybenzene	Faintly positive
Trimethylgallic acid	Strongly positive
Syringic acid	Strongly positive

which were most reminiscent of the deep magenta colour given by hardwoods and isolated hardwood lignin.

In certain cases it was found that a magenta coloration could be produced by reagents other than chlorine and sodium sulphite. For instance it was given by gallic and syringic acids on adding a trace of FeCl$_3$ in the presence of either Na$_2$SO$_3$ or NaHCO$_3$. Gallic acid solutions also give the coloration (1) when aerated in the presence of NaHCO$_3$ and Na$_2$SO$_3$, (2) with a trace of K$_2$CrO$_4$ and Na$_2$SO$_3$, (3) with a trace of CuSO$_4$ and Na$_2$SO$_3$.

In contradistinction to gallic acid, in which the three OH groups are free, and to syringic acid, which has one free OH group, it was found that trimethylgallic acid, which has no free OH groups, could only give the colour reaction with Na$_2$SO$_3$ after previous chlorination. The same has been found to be true of hardwood lignin and, in view of the possible significance of this analogy, the behaviour of pure trimethylgallic acid on chlorination was investigated in detail as follows.

10 g. of the acid were dispersed in 15 ml. of warm water and exposed to a slow stream of chlorine for 30 min. The mixture was cooled and the solid matter (11 g.) was separated by filtration and extracted with light petroleum (B.P. 40–60°). The crystalline substance which settled out from the petrol and was recrystallized from CCl$_4$ consisted of colourless needles (M.P. 120°) and was soluble in EtOH, Me$_2$CO, CHCl$_3$, C$_6$H$_6$ and CCl$_4$. The substance dissolved in NaHCO$_3$ with the evolution of CO$_2$ and its equiv. wt. by titration was 270. (C$_6$Cl$_2$(OCH$_3$)$_3$.COOH requires equiv. wt. 281; C$_6$HCl(OCH$_3$)$_3$.COOH requires equiv. wt. 247·5.) No coloration was given by either FeCl$_3$ or FeFe(CN)$_6$, indicating that the substance contained no phenolic OH groups. It therefore probably consisted of a mixture of mono- and di-chlorotrimethylgallic acids. Contrary to expectation this chlorinated compound, which represented by far the greater part of the original trimethylgallic acid, gave no coloration with sodium sulphite. It was found, however, that the clear aqueous filtrate obtained after the original chlorination did give the coloration. A test for phenols in this filtrate gave a positive result. Repeated chlorination of the already chlorinated trimethylgallic acid gave rise to further small amounts of a phenol which gave an intense magenta coloration with sodium sulphite.

DISCUSSION

It is now realized that the chlorine : sodium sulphite colour reaction is not specific for compounds containing the 1:2:3-trihydroxybenzene nucleus itself since it can also be given by substances in which this nucleus has been modified by substitution. It is worthy of note, however, that so long as at least one OH group remains unsubstituted the colour reaction can be produced by oxidizing agents other than chlorine and weak alkalis other than sodium sulphite. When,

on the other hand, all three OH groups are alkylated chlorination is a necessary antecedent to the production of the magenta coloration with sodium sulphite. In this latter case the major reaction product is a chlorinated derivative of the starting material, but the colour reaction is given by traces of a phenolic compound which is also produced during chlorination. In the case of trimethylgallic acid it must be concluded that chlorine has acted as a demethylating, as well as an oxidizing agent. So far as is known chlorination must always be applied to hardwood lignin before the characteristic coloration is given in the presence of sodium sulphite. By analogy it is therefore strongly suggested that lignin contains a modified pyrogallol nucleus as a recurring unit. This unit may be given the general formula (I).
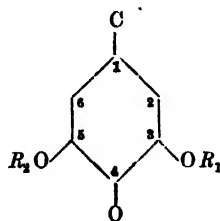
Formula (1)

A magenta colour reaction in the presence of sodium sulphite could be given by such a unit if it were transformed into syringic acid (formula II) or gallic acid (formula III).
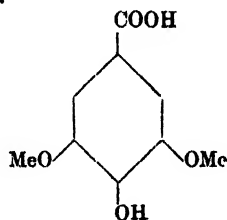
Formula II
syringic acid
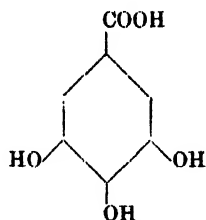
Formula III
gallic acid

In the light of the degradation studies of Freudenberg [1938], Leger & Hibbert [1938] and suggestions in the literature by Klason [1930], Wacek [1930] and others, it is reasonable to assume that R_1 and R_2 in formula (I) are OCH_3 groups and that side chains occur at positions 1 and 4. These side chains could form linkages between essentially similar methylated pyrogallol nuclei.

SUMMARY

1. Continuation of previous work [Campbell *et al.* 1937] indicates that the chlorine : sodium sulphite colour reaction of hardwoods is not specific for compounds containing the 1:2:3-trihydroxybenzene nucleus itself, since it can also be given by substances in which this nucleus is modified by certain substituents.

2. Provided that at least one OH group of a pyrogallol derivative is free the colour reaction is given by oxidizing agents other than chlorine in conjunction with weak alkalis other than Na_2SO_3.

3. If all three OH groups are methylated chlorination is a necessary antecedent to the production of the coloration in the presence of sodium sulphite.

4. The major product of the chlorination of trimethylgallic acid is a crystalline chlorinated derivative of the starting material which gives no coloration with Na$_2$SO$_3$. The coloration is given by a secondary product of chlorination which is shown to be a phenol.

5. The analogy with respect to this colour reaction between certain gallic acid derivatives and hardwood lignin is discussed, and it is suggested that hardwood lignin contains a recurring unit of formula
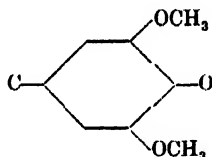
The authors are indebted to the Director of Forest Products Research for permission to communicate these results.

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CCLXXVII. THE BODY FATS
OF SOME SEA-BIRDS

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In earlier papers [e.g. Lovern, 1937] the writer has discussed specific features in
fats which are largely independent of the diet of the animal, and Hilditch &
Lovern [1936] have considered the relationship between species and fat composition. The broad schemes suggested in this second paper are not, however, free
from exceptions, e.g. the whales of the Balaenidae family have fats closely
resembling those of fish to which they are not, of course, zoologically related.
Without, for the present, considering the matter in detail, it is evident that the
diet of marine organisms is ultimately responsible for the fat of these whales
having such a composition.

Sea-birds, living exclusively on fish, should be of interest in this respect. The
fats of comparatively few birds have been examined, and those of only one (the
hen) quantitatively analysed [Hilditch *et al.* 1934]. The hen is particularly
interesting in that its fat contains about 7 % of hexadecenoic acid, until recently
considered characteristic of aquatic life. However, hen fat is not closely similar
to that of fish, since it contains only traces of C_{20} and C_{22} acids. Koyama [1928]
has examined the fats of a number of birds qualitatively, and shown that some
(the carnivorous ones) give small yields of ether-insoluble polybromides. In the
present paper the results of the quantitative analyses of the body fats of four
species of sea-bird are given.

EXPERIMENTAL

The birds were obtained in Shetland waters where it is almost certain that
their diet would consist wholly of fish or other aquatic life. The percentages of
fat recorded are on the plucked, eviscerated and decapitated birds. Small
quantities of accompanying phosphatides (order 10 % of total) were removed by
means of acetone. In Table I are given the particulars of the fats and in Table II
the compositions.

Table I. *Particulars of fats*

Species	No. of specimens	Fat content %	I.V.	Unsaponifiable %
Herring gull (*Larus argentatus*)	7	9·7	107·9	3·0
Skua gull (*Megalestris catarrhactes*)	7	7·0	95·8	6·6
Gannet (*Sula bassana*)	2	6·7	123·9	8·1
Fulmar petrel (*Fulmarus glacialis*)	6	15·2	130·2	7·8

Table II. *Composition of fats (wt. °/₀)*

Species	Saturated				
	C_{10}	C_{14}	C_{16}	C_{18}	C_{20}
L. argentatus	Nil	3·3	18·5	6·2	0·2
M. catarrhactes	Nil	1·9	16·4	5·7	0·2
S. bassana	Trace	3·2	17·1	3·6	Nil
F. glacialis	0·3	2·0	13·9	3·2	Nil

Species	Unsaturated					
	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}	C_{24}
L. argentatus	0·5	4·0 (−2·3 H)	30·5 (−2·8 H)	20·3 (−4·1 H)	16·5 (−4·9 H)	Trace?
M. catarrhactes	0·4	4·6 (−2·1 H)	32·6 (−2·6 H)	19·7 (−3·3 H)	18·5 (−3·8 H)	Trace
S. bassana	1·0	5·2 (−2·0 H)	28·3 (−2·8 H)	24·2 (−4·0 H)	17·4 (−6·0 H)	—
F. glacialis	0·9	3·9 (−2·0 H)	26·9 (−2·8 H)	26·8 (−4·0 H)	22·1 (−6·6 H)	Trace?

The small quantity of acid lower than C_{14} in two of the fats was only examined in the case of the fulmar petrel. It appeared to consist of *n*-decoic acid, no lauric acid being present.

DISCUSSION

It can be seen at once from Table II that all four fats are of the "aquatic" type. By comparison with the frequency curves for marine fish fats [Lovern, 1937] it can be seen that these bird fats are also closely similar to the average marine fish fat. Total C_{16}, C_{20} and C_{22} acid percentages roughly coincide with the maxima on the curves. Total C_{18} is rather high, but on the whole it is obvious that the fats of these birds cannot be very different (as regards the proportions of the various acid groups) from the fat which they ingest.

At the same time the fats have been considerably altered in unsaturation. The stearic acid content is higher than in fish fats, whilst in two cases arachidic acid (which can hardly have been ingested) appears. Concurrently the degrees of average unsaturation of the C_{20} and C_{22} acids are reduced below the average for fish fats. Apparently all the birds have hydrogenated the ingested fat, the process having gone further in the two gulls than in the other two birds. The reason for such hydrogenation may be found in the higher temperature of birds than fish. In the case of fish themselves the author has shown [Lovern, 1938] that a difference of 9° has a slight effect of the type expected.

These birds thus form another exception to the broad rule that fat types can be correlated with phylogenetic relationships. The position may perhaps be summarized thus. Many animals, such as the fish discussed by the writer [Lovern, 1937], produce characteristic depot fats considerably different from the fat which they ingest. The differences are specific and can be correlated with the phylogenetic relationships of the animal in question. Certain other animals (including many fish, such as the cod) deposit fat closely resembling that which they habitually ingest. This may mean one of at least two things: (a) that they have no specific requirements and any type of depot fat will serve equally well, or (b) that in the course of evolution their specific requirements have been produced or modified to suit the normal diet. To decide between these two ideas it would be necessary to make many feeding experiments with different fats and with fat-free diets. Possibly theory (a) would apply to some animals and theory (b) to others.

SUMMARY

The fats of four species of sea-bird have been quantitatively analysed. They markedly resemble the fats of marine fish in the proportions of the various acid groups, although some hydrogenation has occurred.

These birds form another exception to the broad rule that fat types can be correlated with phylogenetic relationships. Two possible explanations are advanced.

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CCLXXVIII. NOTE ON TWO DERIVATIVES OF OESTRONE

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IN connexion with some other investigations we had occasion to prepare *oestrone β-naphthoate* and *oestrone diethylaminoethyl ether*. Neither of these substances appears to have been previously described and we have had them tested for oestrogenic activity. Oestrone α-naphthoate has been prepared by MacCorquodale *et al.* [1936], but no record was made of its oestrogenic activity. Oestrone β-naphthoate resembles the corresponding benzoate [Butenandt, 1930] in that it produces in rats a prolonged period of oestrus although the onset of oestrus is delayed longer than with oestrone itself. The diethylaminoethyl ether of oestrone is of interest as it yields water-soluble salts, but when tested in rats it showed little or no oestrogenic activity.

EXPERIMENTAL

Oestrone β-naphthoate. A solution of oestrone (0·5 g.) and β-naphthoyl chloride (0·6 g.) in pyridine (2·5 ml.) was kept at room temperature for 12 hr. and then heated to 70° for 1 hr. On pouring into a mixture of crushed ice and H_2SO_4 a white solid separated which was collected, washed with aqueous Na_2CO_3, then with water and dried. Extraction with amyl ether gave a small amount of β-naphthoic anhydride and the residue was practically insoluble in diethyl ether. The crude product (0·6 g.) was insoluble in water and glacial acetic acid and very sparingly soluble in alcohol, ethyl acetate and ligroin. Recrystallized from dioxane it had M.P. 262–264°. (Found: C, 81·8; H, 6·4%. $C_{29}H_{28}O_3$ requires C, 82·1; H, 6·6%.)

Biological test. In rats doses of 600 and 100γ produced oestrus after 100 hr. lasting for 10 days; 24, 12, 6γ produced oestrus after 100 hr. lasting for 4 days. The threshold dose appeared to be between 1 and 2·5γ.

Oestrone diethylaminoethyl ether. A mixture of oestrone (50 mg.) diethyl-aminoethyl chloride (120 mg.) and aqueous KOH (25 ml. of 30%) was heated on the water bath for 2 hr., during which time the oestrone slowly dissolved. After cooling and diluting with water the mixture was filtered. The filtrate acidified with HCl gave a precipitate of oestrone (11 mg.) which was collected, and the mother liquor combined with the solution obtained by dissolving the initial filter residue in dilute HCl. The combined acid solutions were again made alkaline and the solid precipitate collected and recrystallized from dilute alcohol. The diethylaminoethyl ether of oestrone (42 mg.) formed glittering leaflets. M.P. 76–77°. (Found: N, 4·0%. $C_{24}H_{37}O_2N$ requires N, 3·8%.) In biological tests it showed no oestrogenic activity in doses of 6, 100 and 1000γ. The base dissolved

in alcohol and treated with ethereal HCl gave the *hydrochloride*, which, crystallized from a mixture of absolute alcohol and ethyl acetate, had M.P. 190–191°.

The biological tests were carried out by Messrs Hoffman-La Roche and Co., Basle, to whom we express our thanks.

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CCLXXIX. THE ORIGIN OF THE PHOSPHORUS COMPOUNDS IN THE EMBRYO OF THE CHICKEN

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SEVERAL of the numerous compounds containing phosphorus present in the embryo of the chicken[1] occur in the yolk and the white of the egg. Those which do are chiefly phosphatides and nucleoproteins but, as Table I shows, other phosphorus compounds also occur in those parts of the egg.

Table I [Plimmer & Scott, 1909]. *Percentage of the total P (94 mg.) at the beginning and the end of incubation of a hen's egg*

	Beginning	End
Inorganic P	Trace	60
Water-soluble P	6·2	8·6
Ether-soluble P	64·8	19·3
Vitellin-P	27·1	0
Nucleoprotein-P	1·9	12

Kugler [1936] has lately found that, on the twentieth day of incubation, i.e. the last day but one, only 25 mg. of the 65 mg. of lipoid P originally present in the yolk remained there; 8 mg. were found in the embryo, and the remainder had been hydrolysed yielding inorganic P. About two-thirds of the phosphatides present were found to be lecithin and one-third kephalin. In view of the large store of phosphatides present in the yolk even shortly before the egg is hatched, we should expect the embryo to avail itself of this store when it needs phosphatides to build up its nervous system and other organs containing these substances. We can test this point by introducing labelled (radioactive) sodium phosphate into the egg before incubation and investigating if and to what extent the phosphatide of the yolk and of the embryo become labelled. If none becomes labelled, we can conclude that the phosphatide molecules in the embryo are not newly synthesized from inorganic phosphate present there; if, however, the yolk phosphatide remains unlabelled while that of embryo becomes radioactive, we can conclude that the phosphatide molecules present in the embryo have not come from the yolk but have been built up in the embryo with the participation of labelled inorganic P. Similar considerations apply to certain other compounds occurring in the embryo.

METHODS

The phosphorus content of a series of solutions is usually determined colorimetrically. For example, the inorganic P present in one sample of an acid-soluble fraction can be determined in this way, and then in another sample the

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[1] A detailed investigation of the acid-soluble phosphorus compounds present in the embryo of the chicken was recently carried out by Needham *et al.* [1937].

phosphagen-P present can be converted into inorganic P, so that colorimetric determination now supplies the value for the inorganic P+phosphagen-P. In our experiments this was inadequate. We had to measure not only the P content but also the activity of the various fractions, so we had to obtain precipitates in each case. To obtain sufficient precipitate when dealing with eggs only incubated for a few days, it was necessary to work with several eggs simultaneously.

We precipitated the phosphorus, after bringing it into the inorganic state, as ammonium magnesium phosphate. The precipitate was then dissolved in 0·1 N HCl and an aliquot part was sucked into a glass cuvette. This was placed below the Geiger counter used to determine the activity of the preparations, while another aliquot part was utilized for the colorimetric determination of the phosphorus content. The glass cuvettes were covered with a thin mica window (5–6 mg. per cm.²) which only absorbed to a negligible extent the β-rays emitted by the radioactive phosphorus; the area of the mica window was 1·1 cm.² and the liquid content of the cuvette amounted to about 0·5 ml. If we attempt to precipitate ammonium magnesium phosphate from the same solution several times, both before and after hydrolysis, large amounts of salts accumulate in the solution and hinder quantitative precipitation.

We were interested in the determination of the activity of 1 mg. P prepared from different phosphorus compounds present in the embryo or in the remains. Accordingly we were not concerned with the quantitative amounts of the P compounds present and so concentrated our efforts on obtaining the various fractions in a pure state—to avoid, for example, traces of inorganic phosphate remaining in the phosphatides extracted from the yolk. As the phosphatides of the yolk were found to be but slightly active, while the inorganic P was strongly active, even a small contamination of the former by the latter was to be avoided. The white, the yolk, the embryo and, in some cases, the amniotic and allantoic liquids were worked up simultaneously.

As regards the white we were only interested in the total activity present after incubation. The white was ignited (reduced to ash) and the phosphorus in it precipitated as ammonium magnesium phosphate.

The yolk was dried with acetone and the phosphatides extracted three times from the dry product with a 3 : 1 alcohol-ether mixture. The alcohol and ether were then evaporated off at about 50° in vacuo and the residue was taken up with light petroleum and filtered. The filtrate was evaporated in vacuo, the residue ignited, and the phosphorus precipitated as ammonium magnesium phosphate.

Another part of the yolk was treated as follows. The acid-soluble compounds were extracted, then the phosphatides were removed as described above, and the residual part containing mainly vitellin-P and nucleoprotein-P was ignited; the P content of this last part was determined as ammonium magnesium phosphate.

The embryos were dropped, immediately after being removed from their eggs, into liquid air and were subsequently pulverized. The embryo powder was then extracted several times with cold trichloroacetic acid—in the first two extractions a 10 % solution was used, and later one of 5 %. The extract was filtered into cold concentrated NaOH solution and divided into three parts, (a), (b) and (c). From (a) a sample of the average acid-soluble P of the embryo was secured, (b) was precipitated with 25 % barium acetate solution at pH 6·5. The cold precipitate was washed with a dilute barium acetate solution, centrifuged and dissolved in a few drops of cold HNO₃. The inorganic P present was then precipitated by adding Fiske's reagent. The remaining filtrate was hydrolysed with N HCl at 100° for 7 min. to split the two labile phosphate radicals of

adenosinetriphosphoric acid. The phosphorus set free was finally precipitated as ammonium magnesium phosphate. Barium hydroxide was added to the filtrate from the barium precipitation to remove any inorganic P, the precipitate was separated by centrifuging and ethyl alcohol was added to the remaining liquid until an alcohol concentration of nearly 60 % was reached. The precipitate obtained after addition of alcohol [Ostern et al., 1936] contained the hexosemonophosphate. Its P content was determined in the usual way. The third part, (c), was hydrolysed with N HCl and 0·1 M ammonium molybdate for 30 min. at 40°. In the course of 30 min. most of the phosphagen present decomposed, so that the inorganic P originally present as such, and that obtained by the decomposition of the phosphagen,[1] were secured together in this fraction.

After removal of the acid-soluble P the embryo was thoroughly treated with an alcohol-ether mixture, as described above, to remove the phosphatides. The residue, containing mainly nucleoprotein-P, was ignited with concentrated sulphuric and nitric acids and the P precipitated in the usual way.

RESULTS

Eggs incubated for 6–18 days. The results of the determination of the specific activities (activities per mg. P) of the different fractions extracted from seven embryos and from the remaining parts of eggs incubated for 11 days are shown in Table II, while Tables III–V give the results obtained with eggs incubated for 18, 16 and 6 days. In addition to the specific activity (activity per mg. P, with that of the P extracted from the white of the egg taken as 100), we have also recorded in Tables II and III the activity (in kicks per minute or in % of amount injected) and the P content of the fraction—this last quantity being determined, in all cases, by the method of Fiske & Subbarow.

Table II. *Specific activity of P extracted from different fractions of an egg incubated for 11 days. (Specific activity of P extracted from the white taken as 100)*

	Fraction	mg. P*	Kicks per min.	Specific activity
Embryo:	Average acid-soluble P	0·074	3·5	59
	Inorganic P	0·077	3·1	51
	Adenosine-P + inorganic P	0·121	6·0	63
	Creatine-P	0·171	8·1	60
	Phosphatide-P	0·561	29·6	67
	Residual ("nucleoprotein") P	1·49	85·6	72
Yolk:	Phosphatide-P	10·4	0·55	0·067

* Indicator mg. P in the sample measured.

Table III. *Specific activity of P extracted from different fractions of an egg incubated for 18 days. (Specific activity of P extracted from the white taken as 100)*

	Fraction	mg. P	% of amount injected	Specific activity
Embryo:	Average acid-soluble P	19·7	53·5	19
	Inorganic (without skeleton) P	10·91	27·2	17
	Tibia and femur-P	4·50	7·6	11
	Adenosine-P	0·048	0·14	20
	Phosphatide-P	1·08	1·7	11
	Residual ("nucleoprotein") P	0·204	0·3	10
Yolk:	Acid-soluble P	0·828	1·3	11
	Phosphatide-P	17·50	0·28	0 11
	Residual P	2·16	0·12	0·40

[1] On the phosphagen content of the embryo of the chicken, cp. Lehmann & Needham [1937].

The figures for the specific activities (activities per mg. P) of different frac tions extracted from an embryo and from the remaining parts of an egg incubated for 18 days are shown in Table III. The P content in mg., the percentage of the injected activity present in the fraction and the relative specific activity are recorded; the specific activity of the P extracted from the white of the egg is taken as 100.

The specific activities obtained when the eggs were incubated for 16 and 6 days respectively are seen in Tables IV and V.

Table IV. *Specific activity of P extracted from different fractions of an egg incubated for 16 days. (The specific activity of P extracted from the white taken as 100)*

	Fraction	Specific activity
Embryo:	Average acid-soluble P	14
	Inorganic (without skeleton) P	14
	Tibia and femur-P	15
	Creatine-P	14
	Hexosemonophosphate-P	19
	Phosphatide-P	12
	Residual ("nucleoprotein") P	16
Yolk:	Acid-soluble P	12
	Phosphatide-P	0·14
	Residual P	1·22

Table V. *Specific activity of P extracted from different fractions of 10 eggs incubated for 6 days. (Specific activity of embryo phosphatide P taken as 100)*

	Fraction	Specific activity
Embryo:	Phosphatide P	100
	Average (phosphatide) P	113
Yolk:	Inorganic P	60
	Acid-soluble minus inorganic P	34
	Phosphatide P	0·032
	Residual P	1·3

As the figures show, the phosphatides extracted from the yolk are only slightly active, while those extracted from the embryo show strong activity; 1 mg. of embryo phosphatide-P is at least 100 times as active as 1 mg. yolk phosphatide P. Furthermore, the specific activity of the embryo phosphatide-P is about as high as that of the embryo inorganic P, showing that an inorganic P atom reaching the embryo has about the same chance of entering the skeleton as of being incorporated in a phosphatide molecule by an enzymic process—which of the two systems it enters is governed solely by probability considerations. From this it follows that the phosphatide molecules in the embryo are not identical with those derived from the yolk, but are synthesized in the embryo.

The formation of labelled phosphatides in growing eggs was investigated by Hevesy & Hahn [1938]. It was found that the phosphatides present in the yolk are taken up from the plasma by the ovary and incorporated into the latter; as soon as the yolk leaves the ovary no more change occurs in the content or com position of its phosphatides. When labelled phosphate is administered to a hen after the yolk has left the ovary and is located in the oviduct, the egg takes up active phosphate but no active phosphatide is formed. In experiments *in vitro* as well, eggs placed in radioactive sodium phosphate solution take up active phosphate but no active phosphatides are formed. The slight activity of the phosphatides present in the yolk of incubated eggs is presumably due to the

influx into the yolk of small amounts of active phosphatides synthesized in the embryo. This view is supported by the fact that the ratio of the specific activities of the embryo phosphatide-P and yolk phosphatide-P was much larger (3000) in the 6 days experiment than in the 18 days experiment (100). The activity of the residual P of the yolk, which is mainly composed of vitellin and nucleoprotein, was larger than that of the phosphatides; this can be understood if we admit the possibility that the extraction of the strongly active, non-protein constituent of the yolk is not quantitative, for in this case the specific activity of the residual P would be increased.

The embryonic residue obtained after extraction of the acid-soluble and ether-soluble constituents is composed chiefly of nucleoproteins. That the specific activity of the nucleoprotein-P is the same as that of the inorganic P extracted from the embryo is not surprising, because much less nucleoprotein is present in the yolk than in the embryo (Table I). The greater part of the nucleoproteins present in the embryo must therefore have been built up in the course of incubation; during this process labelled phosphate has an opportunity of entering the nucleoprotein molecules.

The radioactive sodium phosphate of negligible weight injected into the white of the egg labels the inorganic P present in the latter. The labelled inorganic P is transported from place to place, along with the other water-soluble phosphorus compounds present in the white and the yolk, and becomes partly incorporated in the embryo. The inorganic P so removed is replaced by some formed by hydrolysis, mainly of phosphatides and vitellin. In this way the radioactive inorganic P injected into the egg becomes more and more diluted with inactive inorganic P and its specific activity (activity per mg. P) diminishes accordingly. Whereas at the start of the experiment only traces of inorganic P are present in the egg, at the end of the incubation about 60 mg. are found, mostly in the skeletal part of the embryo. As the inorganic P used in the synthesis of the different P compounds present in the embryo becomes less and less active in the course of incubation, we should expect to find the phosphorus compounds synthesized at a later date much less active than those built up at an early stage in the incubation. This does not, however, seem to be the case. Actually, we have not yet compared the activity of, for example, the phosphatides extracted from the nervous system, which is built up at an early stage, with that of the phosphatides extracted from the skeleton, which is synthesized at a late stage of development. But the fact that the specific activities of the inorganic P, the phosphatide-P and the nucleoprotein-P were found to be equal to within the errors of the experiment suggests that no large differences in the specific activities of phosphorus compounds formed at different dates can be expected, though minor differences could possibly be found. This must be interpreted as being due to the ceaseless breaking-up and the rebuilding of the molecules in the embryo under enzymic action, a process which leads to equipartition of the activity between the different phosphorus molecules.

We should most expect to find phosphate layers of different specific activity in the skeleton. The rate of phosphorus exchange in the bone tissue is a comparatively slow process, though the atoms of the embryonic bone tissue may be comparatively easily replaceable. A difference in the specific activities of inorganic P extracted from bone tissue (tibia+femur) and from the other organs of the embryo is shown in Tables III and IV, which is, however, to be interpreted cautiously.

Distribution of radioactive phosphate in the egg

The greater part of the sodium phosphate injected into the white is still found at the end of the experiment in that part of the egg. The distribution of the activity between white, yolk, connecting fluids (which were not, however, free from white and yolk) and embryo is seen in Table VI.

Table VI. *Distribution of injected active phosphate between different parts of the egg*

Time of incubation	Fraction	% activity
6 days	White	61·6
	Yolk	10·3
	Liquids	26·0
	Embryo	1·7
18 days	White	14·9
	Yolk	1·7
	Liquids	19·8
	Embryo	63·0

The low activity of the yolk might possibly be due to a slow rate of penetration of the vitellin membrane by the phosphate ions; this point is under investigation. Another possible explanation is that the inorganic P content of the yolk is lower than that of the white. If a distribution equilibrium is reached, the activity should be proportional to the amount of inorganic phosphate present in the phase in question, since the inorganic P, among all the P compounds present in the yolk and white, is practically the only source of activity; in the 6 days experiment, for example, 10 % of the 10·3 % activity found in the yolk was present as inorganic P. Finally we have to envisage the possibility that a part of the inorganic phosphate injected is not freely movable in the white—it might be precipitated as calcium phosphate or attached to proteins, its mobility being lowered thereby.

We have also carried out experiments in which 0·1 ml. physiological NaCl solution containing a negligible amount of labelled sodium phosphate was injected into eggs which were not incubated. After the lapse of 5 days the distribution of the activity in different parts of the egg was determined; 97 % was found in the white and 3 % in the yolk. As was of course to be expected, a still greater preference for the white was shown by the active phosphorus in this experiment; the duration of the experiment was shorter than that of those discussed above, and transport of phosphorus from the white to the embryo was absent.

To test whether the water injected encountered any hindrance in its propagation through the egg, we injected 0·2 ml. heavy water into the white of the egg; after the lapse of 5 days water was distilled separately from the white and from the yolk and the densities determined. We are much indebted to Mr O. Jacobson for carrying out the density determinations using Linderström-Lang's float method. He found that the water prepared from the white had a density exceeding that of normal water by 484 parts per million, while the corresponding figure for the water obtained from the yolk was 437. The deuterium content of the water distilled off from the yolk was thus found to be only about 10 % lower than that of the water from the white, showing that in the course of 5 days the water injected was very nearly evenly distributed throughout the egg, in contrast to the injected active phosphate. The anomalous behaviour of the latter, while of interest in the study of the circulation of phosphate ions in white and yolk, in

no way influences the investigation of the main problem discussed in this paper—namely, if and to what extent the molecules of the different phosphorus compounds present in the embryo are built up there or drawn, ready made, from the yolk.

Introduction of labelled hexosemonophosphate into the egg to be incubated

In one set of experiments, instead of following up the fate of labelled inorganic P in incubated eggs, we introduced radioactive hexosemonophosphate. Prof. Parnas very kindly presented us with this radical (prepared by Dr Ostern) in the form of barium hexosemonophosphate, from which, by treatment with sodium sulphate in the cold, the sodium compound of the ester was obtained. 0·2 ml., containing about 0·2 mg. P as hexosemonophosphate salt and about 3 mg. sodium sulphate, was injected into the white of each of the eggs to be incubated; to avoid decomposition of the ester, the solution was kept ice-cooled until it was injected into the egg. Of the 10 eggs receiving this treatment, only two supplied living embryos. After a lapse of 14 days, 7·7 % of the activity injected was found to have been incorporated in the embryo (5·8 % in the yolk) and a large fraction was also to be found in the white and in the connecting liquids. If, of the various fractions extracted from the embryo, we had only found activity in the fraction containing hexosemonophosphate, we should have had to conclude that the hexosemonophosphate does not decompose in the egg but enters the embryo as such. In view of the results obtained in the experiments carried out with labelled inorganic phosphate, however, such behaviour was hardly to be expected. Furthermore, Kay [1926] found that in the embryo the phosphatase activity of the developing bone was extremely high, the phosphatase decomposing the hexosemonophosphate. We isolated the hexosemonophosphate from the embryo, as described on p. 2148, and compared the specific activity of this fraction with that of the inorganic phosphate (+creatine-P). We also isolated the phosphatide fraction and the residual phosphorus fraction containing mainly nucleoprotein-P. As Table VII shows, no conspicuous difference can be seen between the specific activities of the different fractions of the embryo, with the possible exception of the residual P. In these experiments small activities had to be measured and the differences found between the first three fractions lie within the errors of the experiment. The results obtained suggest the explanation that active inorganic P splits off from the labelled hexosemonophosphate injected and is incorporated in the different phosphorus compounds of the embryo, while the hexosemonophosphate molecules extracted from the embryo are not those synthesized by Dr Ostern but are molecules built up by the chicken's embryo.

Table VII. *Specific activity of P from different fractions extracted from two eggs incubated for 14 days after the injection of radioactive hexosemonophosphate. (Specific activity of P extracted from the white taken as 100)*

	Fraction	Specific activity
Embryo:	Inorganic P	24
	Hexosemonophosphate-P	26
	Phosphatide-P	20
	Residual ("nucleoprotein") P	11
Yolk:	Inorganic P	36
	Hexosemonophosphate-P	18
	Phosphatide + residual P	0

The low value found for the residual P of the embryo may possibly be due to the building up of a part of the nucleoprotein fraction at an early date before much of the active hexosemonophosphate introduced has decomposed. The phosphatide-P and residual P extracted from the yolk were found to be inactive. These fractions were found to be only slightly active even after the injection of strongly active inorganic P, and the absence of activity after the injection into the egg of the much weaker hexosemonophosphate was only to be expected. The hexosemonophosphate fraction isolated from the yolk had a specific activity of 18; the inorganic P, 36. The larger value found for the specific activity of the inorganic P is possibly to be explained in the following way. Some active hexosemonophosphate diffuses into the yolk and partly decomposes into active inorganic P; this is the source of most of the active inorganic P which we isolated from the yolk. The hexosemonophosphate, isolated by the method outlined on p. 2149, contains, besides the active hexosemonophosphate, some non-active hexosemonophosphate and possibly also some other acid-soluble P compound separated simultaneously, which diminishes the specific activity of the "hexose-monophosphate" fraction isolated from the yolk. In the embryo, on account of the strong enzymic action prevailing there, all phosphorus compounds become activated; on the other hand, in the yolk, as we have just mentioned, no such activation takes place.

On the phosphatide synthesis in the embryo of the chicken

We saw that the phosphatide molecules present in the chicken's embryo are not identical with those formerly located in the yolk, but that they were synthesized in the embryo. The work of Schönheimer & Rittenberg [1936] gives us important information about the units which are utilized in the synthesis. They found, by making use of deuterium as an indicator, that the developing hen's egg forms no new fatty acids and their result excluded also the possibility that unsaturated fatty acids present in the egg had been hydrogenated during development. Needham [1931], on the other hand, found that a marked desaturation occurs in an aqueous emulsion of embryonic tissues mixed with the corresponding yolk and vigorously shaken. The embryo must thus make use of the fatty acids present in the yolk to build up its phosphatides; in doing this it possibly gives some preference to the less saturated fatty acids. The fatty acid components of the phosphatides extracted from the embryo are found to be less saturated than those extracted from the yolk residue. The at first sight puzzling fact that the embryo, instead of using the phosphatide molecules found in great abundance in the yolk, synthesizes its own phosphatide molecules, becomes less puzzling when we envisage the likely possibility that the synthesis of phosphatide molecules is also a step in other chemical processes, which occur simultaneously in the growing embryo.

SUMMARY

Radioactive sodium phosphate was injected into hen's eggs which were then incubated in some experiments for 6, and in others for 11, 16 and 18 days. While the phosphatide-phosphorus extracted from the embryo always showed a high specific activity (activity per mg. P), that extracted from the yolk was hardly active at all. The phosphatide molecules present in the embryo could not therefore have been taken from the yolk only, but must have been synthesized in the embryo. The investigation of the "acid-soluble" and residual (mainly nucleoprotein) phosphorus extracted from the embryo led to a similar

result—namely, that the ratio in which the labelled inorganic phosphorus atoms are incorporated into the different phosphorus compounds present in the embryo is governed solely by probability considerations. Practically all the phosphorus atoms present in the various compounds of the embryo must pass through the stage of inorganic P: only the inorganic phosphorus present in the embryo is taken as such from the yolk or the white.

In some experiments, instead of radioactive sodium phosphate, labelled hexosemonophosphate was injected into the egg before incubation. The hexosemonophosphate-phosphorus extracted from the embryo had about the same specific activity as the inorganic and the phosphatide phosphorus extracted. This result suggests that inorganic phosphate radicals which have split off from the hexosemonophosphate and from other compounds present in the yolk and the white, rather than the hexosemonophosphate molecules introduced into the latter, are utilized to build up the phosphorus compounds of the chicken's embryo.

We wish to express our best thanks to Prof. N. Bohr for the numerous facilities kindly placed at our disposal and to the Danish State Farm at Hilleröd for incubating many of the eggs investigated.

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CCLXXX. THE VITAMIN B₁ CONTENT OF FOODS
II. ADDITIONAL VALUES

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WE have previously published the vitamin B₁ values of about 80 foods [Baker & Wright, 1935]. The following additional values have been obtained by the same technique. A few figures for breads, some of which have been published elsewhere [Baker *et al.* 1937], are included for convenience of reference. Values are given in terms of International units.

Material	Dose (g.)	I.U./g.	Remarks
		Meat, offal and fish	
Raw lean beef	4	0·3 –0·5	0·5 previously published
	6	0·4	
Cooked tongue	4	0·3 –0·45	—
Stewed steak	6	0·1 –0·2	—
Stewed rabbit	4	0·2 –0·4 ⎱	Same sample
	6	0·2 ⎰	
Stewed tripe	6	Less than 0·2	—
Fried sweetbread	4	0·2–0·4	—
Fried bacon	0·5	2·8 –4·8	Cf. roast pork 3·2, boiled ham 2·2
Fried herring	4	0	—
		Dairy produce	
Sweetened condensed milk	5	0·4 –0·6	Cf. raw milk 0·23
		Vegetables	
Cooked haricot beans	4	0·4 –0·6 ⎱	Cf. raw 1·2
	6	0·35–0·6 ⎰	
Cooked butter beans	4	0·2 –0·3	—
Raw green runner beans	4	0·25–0·75	—
Raw cauliflower	4	0·4 –0·5	Previous value 1·1
Raw marrow	8	0·1 –0·2	—
Raw fresh peas	1	1·6 –2·8	—
Cooked fresh peas	4	0·8 –1·1	Same sample
	2	1·0 –1·2	
Raw dried peas	1	4·2 –4·8	Cf. canned peas 1·2
Cooked dried peas	3	0·15–0·45	—
	5	0·2 –0·4	
Stewed onions	4	0·3 –0·5	Cf. raw 0·4
		Fruits	
Black currants	5	0·0 –0·2	—
Blackberries	5	0·0 –0·2	—
Melon	10	0·1 –0·2	—
Fresh ripe peach	4	0·1 –0·4	—
Canned peach	5	0·1 –0·3	—
Syrup from peach	—	Traces only	—
Raspberries	6	0·25–0·35	—
Red currants	5	0·1 –0·2	—
Strawberries	4	Traces only	—
		Cereals	
Boiled rice	8	—	—
Ground rice	8	0·15–0·35	—
Sago	8	—	—
Tapioca	8	—	—

Material	Dose (g.)	I.U./g.	Remarks
		Cereals (*cont.*)	
Semolina (1)	8	0·35–0·45	2 different samples
(2)	4	0·55–0·65	
Millet (Juba)	3	0·6 –1·0	—
	2	0·8 –1·2	
Oatmeal	1	1·1 –1·7	Previous figures 3·25, 1·4
	2	1·0 –1·2	
Rolled oats	1	1·8 –2·7	—
	2	1·45–2·0	
		Miscellaneous	
Black treacle W. Indian	1	—	—
		Breads	
Special brown (London):			
Sample (1)	3	0·45–0·6	Cf. previous values of other brown breads, 0·5, 0·7, 0·5
Sample (2)	3	0·6 –1·0	Cf. previous values for wholemeal bread, 1·1, 1·2, 1·2
Special brown (Cheshire)	3	1·2 –1·4	Probably in the germ bread class. Cf. previous values of other germ breads, 1·2, 1·65, 1·7
Wholemeal stone-ground (Cambridge)	4	0·4 –0·6	A bread definitely lower in vit. B_1 than others known to be wholemeal
	3	0·35–0·45	
Austrian, Sample (1)	3	0·5 –0·7	Cf. also previously published values for white bread; 4 samples London 0·14, 0·14, 0·15, 2·0; 1 sample Scotland 0·24; white with malt, 2 samples, 0·35, 0·35
Sample (2)	3	0·5 –0·9	

These foods have all been assayed by the rat-bradycardia method of Birch & Harris [1934], and since this method has been in use in this laboratory for 4 years a few comments may be useful.

Values obtained are in good agreement with those published by other workers, e.g.:

Material	Value by brady-cardia method I.U./g.	Values reported by other workers, I.U./g.
White bread	0·14–0·2	0·12–0·15 [Harris, 1937]
		0·17–0·25 [Copping & Roscoe, 1937]
Brown bread	0·5 –0·7	0·6 [Morgan & Frederick, 1935, 1]
Whole wheat bread	1·15, 1·2	1·17, 1·3 [Morgan & Frederick, 1935, 1]
Wheat, whole grain	1·2 –3·4	1·5 [Scheunert & Schieblich, 1937]
Bran	1·3, 3·0	3·6 [Leong & Harris, 1937]
Wheat germ, raw	4–22 (118 samples)	7·6–9·4 [Morgan & Frederick, 1935, 2]
Brewer's yeast	6·0 –23	16–18 [Chick & Jackson, 1932]
		18–20 [Birch & Harris, 1934]
Banana	0·5	0·3–0·6 [Van Veen, 1935]
Potato	0·4	0·3–0·6 [Van Veen, 1935]
Lean beef	0·5	0·3–1·0 [Van Veen, 1935]
Raisins	0·75	0·5 [Morgan *et al.* 1935]

Assays repeated after long intervals have been found to give consistent results, e.g.:

	I.U./g.
White bread, sample (1) assayed 1935	0·0–0·2
(2) assayed 1936	0·1–0·2
(3) assayed 1937	0·1–0·3
Whole wheat, 1 sample, 1st assay 1935	1·6–2·4
2nd assay 1936	1·9–2·1

Recently Robertson & Doyle [1937] have called attention to various difficulties in the use of the method. They find wide differences in the rats' heart rate as recorded at two portions of the same electrocardiographic tracing and also occurring after a short interval without dosage of any vitamin B_1. As the result
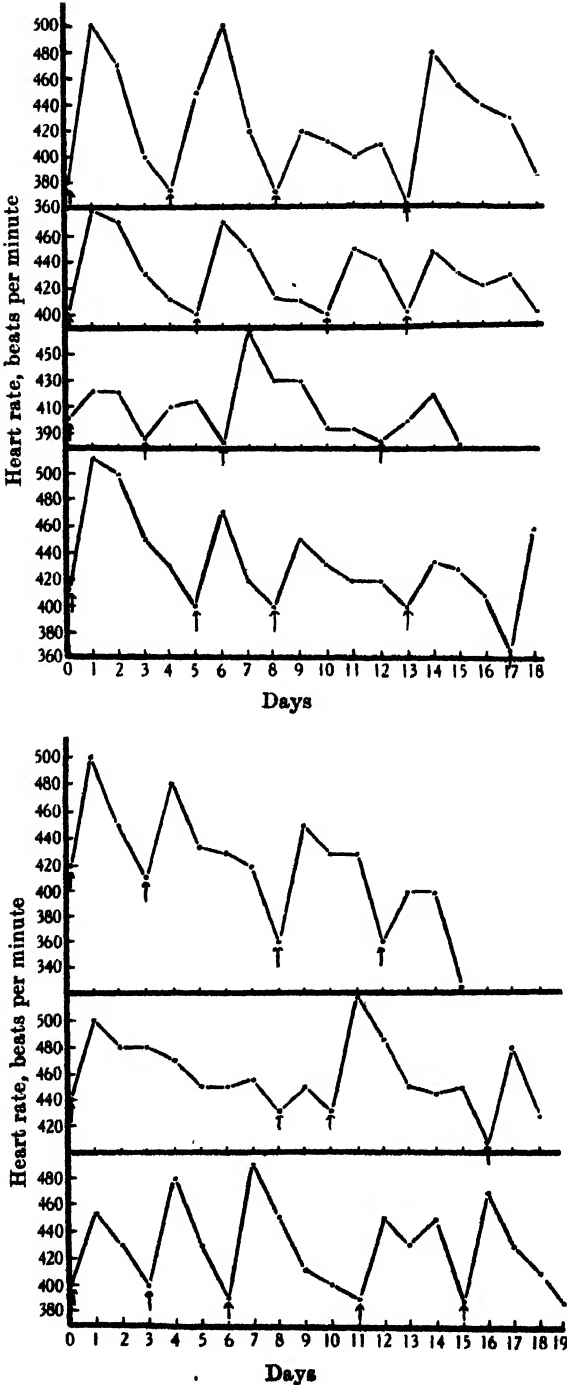
Fig. 1. Heart rates recorded daily. Seven rats taken consecutively from the record book.

of experience involving over 80,000 readings, we do not find these difficulties serious. Irregularities such as extra systoles and dropped beats occur sometimes, though not often, and in these cases it is important to note that it is the rhythm which is being counted, not the actual number of beats. In electrocardiographic tracings 8–10 in. long, we do not find a variation greater than 5–10 beats per min., representing 1–3 % of the total heart rate and only a fraction of the variation which is brought about by an increased intake of vitamin B_1.

The accompanying graphs (Fig. 1) are records of the behaviour of a group of 7 consecutive rats taken from the daily record book. These show that the heart rate after a dose—as seen in 24-hourly readings—rises steeply and gradually drops to the initial level.
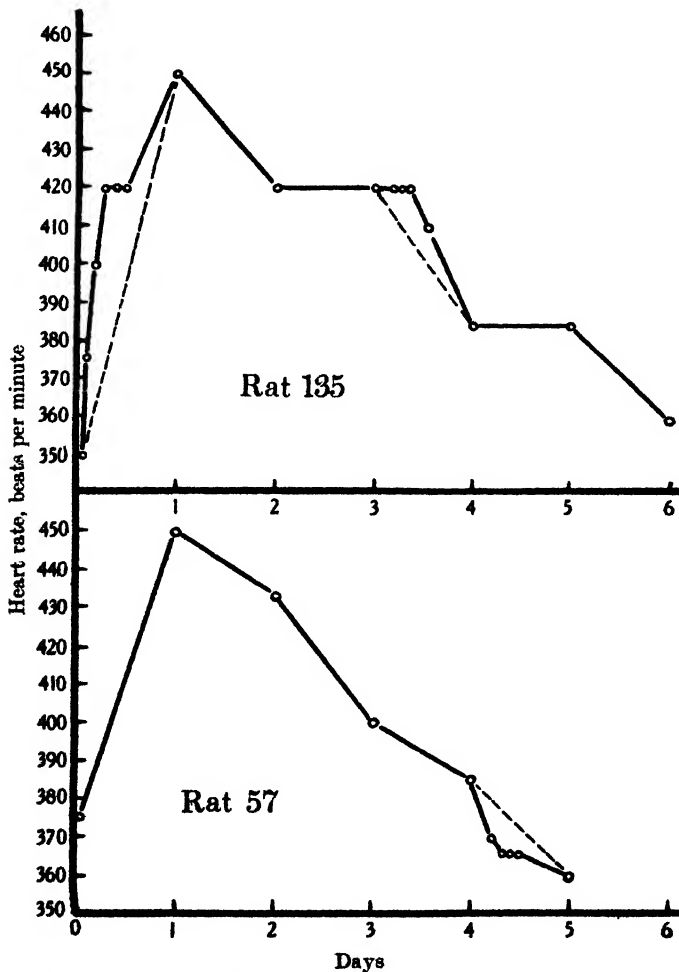
Fig. 2. Records of the heart rates of 2 rats measured 24-hourly and also at more frequent intervals to show the progressive rise or fall in heart rate. ——o——o—— All readings. — — — 24-hour readings.

The records of rats nos. 135 and 57 (Fig. 2) show further that readings taken at more frequent intervals fall into place with the 24-hourly ones.

The rats' heart rate is, however, sensitive to very small variations in vitamin B_1 intake, and, while this is an advantage in permitting the assay of substances containing traces of the vitamin, it necessitates stringent care in practice to keep a continuous check on the basal diet by having 2 or 3 animals always as negative controls. That this is necessary is shown by the fact that a yeast sold commercially in America for use in vitamin B_1-deficient diets, and guaranteed to be free from vitamin B_1, was found electrocardiographically to contain about 1 I.U. vitamin B_1 per g. This amount in the yeast of a basal diet had actually been the cause of some puzzling results which were later explained when the presence of vitamin B_1 was known. On a basal diet completely devoid of vitamin B_1, the heart rate of a rat, if already dropping, becomes slower every day until death occurs with a rate usually of less than 300 beats per min. (see negative control graph, Fig. 3).
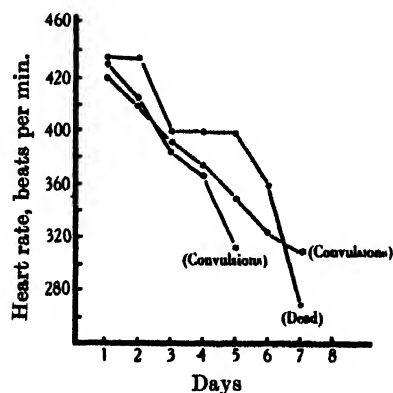
Fig. 3.

Fig. 4.

Fig. 3. Negative control records.
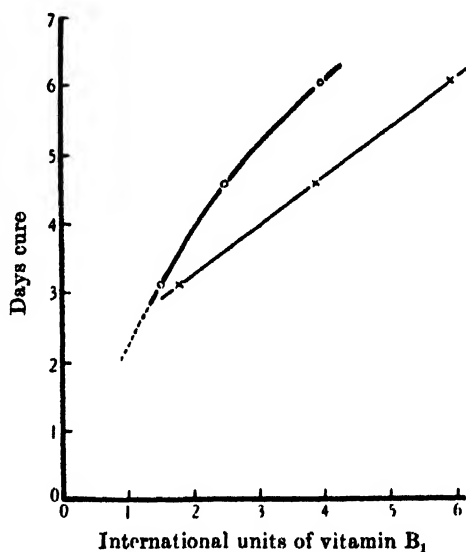
Fig. 4. The cardiographic response of the rat's heart to International Standard vitamin B_1. Composite curve from over 260 readings at each of 3 dose levels. o——o Duration of cure/dose. ×——× Duration of cure/log dose.

15 mg. Int. St.	25 mg. Int. St.	40 mg. Int. St.
3·1 days (283 readings)	4·6 days (280 readings)	6·0 days (260 readings)

As in all biological assays, it is necessary to test the unknowns simultaneously with the standard of reference. In this laboratory three levels of international standard have been fed weekly and from the results obtained a considerable amount of information has been collected concerning the response to this substance. The curve shown in Fig. 4 was obtained from over 260 readings at each level and is logarithmic in form. There is little difference between the curves obtained from weekly readings and this composite curve, a fact which emphasizes the specificity of the cardiac response to vitamin B_1 and its independence of disturbing factors. '

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CCLXXXI. STUDIES OF THE ESSENTIAL UNSATURATED FATTY ACIDS IN THEIR RELATION TO THE FAT-DEFICIENCY DISEASE OF RATS

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THE experiments of Burr & Burr [1929; 1930] and of Burr *et al.* [1932] clearly established that the complete exclusion of fat from a diet containing all known accessory factors slowly produced in young rats a definite disease. Its symptoms were cessation of increase in length and weight with development of a dry scurfy skin, scaly tail, irregular ovulation and lesions of the kidney and urinary tract. The rats receiving the fat-free diet ate as much food and drank twice as much water as their controls, but notwithstanding became extremely emaciated. At 4 months after weaning their weight reached a level of about 150 g. and then remained stationary, but weight increase was resumed when 10 drops daily of the fatty acids from lard were added to the diet. The curative effect of the dose was immediate and the gain in weight was quite disproportionate to the caloric value of the curative material administered. Various other oils were tested and the conclusion was drawn that their curative potencies appeared to be correlated with their linoleic acid contents. Burr and Burr therefore suggested that the rat is unable to synthesize linoleic acid and that this acid is essential for its normal growth. Linolenic acid was also examined and was reported to be equal to linoleic acid in curative effect. The full literature of this subject has recently been reviewed by Anderson & Williams [1937] to whose excellent account the reader is referred. Work which is particularly relevant to the present study will be referred to as the various materials tested are discussed.

There is no evidence as to the part played in metabolism by these unsaturated fatty acids. Linoleic acid is present chiefly in the fatty acids of the phospholipins and to a less extent in the neutral fat. Linolenic acid, on the other hand, appears to be changed immediately on entering the body and is not found even in small amounts, unless when large quantities have been administered. It is known, owing chiefly to the work of Sinclair [1929–30], that the highly unsaturated acids are held in the phospholipins with extreme tenacity and are eliminated only very slowly when animals are transferred to a diet from which the acids are absent. They do not appear to represent stages in the combustion of the fatty acids, but to have some unknown significance in the body metabolism. It seemed possible that an oxidation product might be formed as a further stage in some metabolic process and it appeared desirable to compare the effect of the unsaturated acids with that of some closely related oxidation products.

The present investigation has, therefore, included the following:

(1) A reproduction of the work of Burr and Burr with a study, as far as possible quantitative, of the relative potencies of linseed oil, linoleic and linolenic

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acids, both to restore weight increase and to alleviate skin lesions: a test of the potencies of the elaidized linoleic and linolenic acids compared with those of the original products.

(2) A test of the potencies of certain oxidation products of linoleic and linolenic acids.

(3) A comparison of the activities of fractions of the unsaturated acids from lard and from linseed oil.

(4) A comparison of the activities of linseed oil and raisin seed oil.

(5) An examination of the potency of the methyl ester of the highly unsaturated docosahexaenoic acid ($C_{22}H_{32}O_2$) of cod liver oil. Through the generosity of Dr E. H. Farmer an opportunity was afforded to test the efficacy of this acid recently isolated by him from cod liver oil [Farmer & Van den Heuvel, 1938, 2].

(6) A test of the curative actions, if any, of chaulmoogra oil and chaulmoogric acid, of methyl arachidate and of 9:10:12-trihydroxystearic acid.

BIOLOGICAL TECHNIQUE

The experiments described in the present paper have been in progress for about 5 years so that the technique has varied slightly over this period, but the regime of Burr et al. [1932] has been followed fairly closely.

Young rats weaned on a stock diet at an age of 21 days and a weight of about 40 g. were placed in separate cages with coarse wire grids. Sometimes fine wire grids and cotton-wool bedding were given when the animals seemed in a precarious condition in severe weather. As it takes months to prepare the animals it is very important not to lose them when the time for testing approaches.

The diet consisted of: casein (British Drug Houses, fat- and vitamin-free), 12%; sucrose, 84·1%; salt mixture, McCollum 185 [McCollum & Davis, 1914], 3·9%.

The diet was given ad libitum, slightly moistened. The vitamin B complex was supplied as 0·65 g. per head daily of dried brewer's yeast which had been continuously extracted with ether for 24 hr. This supplement was given separately and was frequently refused at first, but if it was withheld for the first 7–10 days it was subsequently taken with avidity and was found an admirable vehicle for administering the test dose, whether this took the form of an oil or a solid. Vitamins A and D were given as 0·5 mg. daily per head of the unsaponifiable fraction of a cod liver oil concentrate, of value 4000 blue units, dissolved in paraffin oil. In the latest experiments (rats belonging to litters 4273–4350) vitamin A was derived from a fish liver oil concentrate, but vitamin D was given as 2–3 I.U. daily of irradiated ergosterol dissolved as before in paraffin oil. Vitamin E was not given except in the latest experiments (rats numbered as above) when it was given for a portion of the depletion period and for the test period as an unsaponifiable fraction of wheat germ oil, prepared by the Glaxo Laboratories. It was administered without solvent in a dose equivalent to about 2·0 g. wheat germ oil weekly. The distilled water used for drinking and for moistening the food contained 0·27 mg. KI per l.

On this diet the behaviour of the rats conformed with that described by Burr & Burr. After about 2 months the weight curve began to flatten and the skin lesions began to appear and to become progressively more severe. Tests were usually started after about 4 months on the diet, but no rigid rule was established in this respect. A few tests which were started when the rats had only been receiving the deficient diet for about 70 days could not be regarded as reliable, at

least from the standpoint of resumption of weight increase. After a period of 4 months or more on the deficient diet the writers considered that weight increase could be accepted as some measure of the potency of the materials administered and the total weight increase in an experimental period of 35 days was taken as one criterion. The total weight increase was calculated as the weight on the day on which dosing was begun subtracted from the weight 35 days later.

For quantitative estimation, weight increase has been the chief criterion used by Burr and his co-workers, by Moore [1937] and by Turpeinen [1938]. The last also used as criterion the restoration of the oestrous cycle towards normal.

While accepting weight increase as one criterion, the present workers hold very strongly the opinion that, since weight increase is a non-specific response, it should not be used as the only criterion, and that cure of symptoms must be included as an indispensable part of the test. A serious objection to the use of this additional criterion is the great irregularity with which most of the lesions tend to appear. The kidney symptoms described by Burr & Burr were sometimes seen. The development of the so-called scaly tail, better described as a corrugated or annulated tail, was very irregular, the tails of individual rats sometimes being almost normal at a time when their litter-mates were showing a very severe degree of annulation. The other symptoms observed were general dryness of the skin and thinness of the hair with much scurf, showing best on parts of the body where the fur was dark. Dryness and scurfiness of the fore and hind paws and ears were also present; the most regularly observed site for this state was the dorsal surface of the hind feet and front of the ankles. Dryness here occurred invariably and was finally adopted as the most satisfactory criterion for studying the cure of skin symptoms. With potently curative materials the skin of the insteps could be restored to normal within the 35 days of the experimental period during which curative material was administered and weight increase measured. Only three degrees of healing could be satisfactorily distinguished: complete healing, designated by + + in the Tables, in which the ankles were restored to normal; partial healing, designated by +, in which improvement was definitely present but the ankles were still abnormal; no healing, designated by θ, in which the ankles were unchanged or worse.

Even when lesions of the tail were well developed, as they usually, but not always, were, it was found impossible to use them to measure the rate of healing since the process was much too slow. Within the experimental period of 35 days, even when healing of the ankles was complete, the tail might show little change. If the cure was continued long enough the tail could be restored entirely to normal, but in 35 days it might even appear worse owing to the vigorous desquamation which sets in at the beginning of the healing process. The writers share the impression expressed by Brown & Burr [1936] that the skin lesions are somewhat influenced by weather changes, being aggravated by the same type of cold dry weather as causes the human skin to chap. This adds to the difficulties in appraising cure. Very severe lesions of the external skin about the mouth of the rats were sometimes observed but these were due to the burning action of the curative materials administered and not to the deficiency. For instance, methyl linoleate and linolenate, and the methyl docosahexaenoate in these experiments, and ethyl laurate and acids from hydrogenated tung oil in other experiments, had the effect of seriously damaging the skin about the mouth, causing the upper layers with all the hair attached to shell off. No internal lesions appeared to have been produced. Materials having this effect were therefore always intimately mixed with the dried yeast dose, and in this way did not wet the rat's lips and were not objected to.

Although a source of vitamin E was given in some experiments and not in others there was no convincing evidence that its absence made any significant difference in this test. Skin lesions were healed and weight increase appeared to be promoted as well in its presence as in its absence. A quite different type of skin lesion with large raw and bleeding areas appeared in some cases and may have been due to vitamin E deficiency but this is quite uncertain. All the rats tended to become very nervous and fidgety when untreated and to improve on treatment, so that the essential unsaturated fatty acid was the probable sedative factor.

Since there is no recognized standard material and no curve of response available for tests on the unsaturated fatty acids, estimations of the potency of materials were made as a series of simultaneous comparisons in which the weight increase and degree of healing of the skin of the ankles of litter-mate rats were compared. Some negative controls and some positive controls receiving linseed oil were usually included, but the larger number of rats was devoted to some special comparison. Thus, for example, in the first experiment the action of methyl linoleate was directly compared with that of methyl linolenate; in the second the potencies of linusic and isolinusic acids were compared with one another and with that of linseed oil. When only small amounts of material were available or when preliminary experiments gave an unequivocally negative result, full comparisons were not made. A certain number of materials tested in this way are included in Table III.

Since rats for this test take so long to prepare they were sometimes used a second time when the material first tested had proved negative. Negative controls were also subsequently used for tests. As an additional form of positive control, rats which had given a negative result were usually eventually treated with some known positive material such as wheat germ oil or linseed oil, but no record of this form of positive control is included here.

EXPERIMENTAL

(1) *Comparison of the relative potencies of methyl linoleate, methyl linolenate and linseed oil with one test of methyl β-linoleate. Effect of elaidization on the potency of linoleic and linolenic acids.*

Burr *et al.* [1932] found methyl linoleate and methyl linolenate equally effective in curing the lesions and restoring weight increase in rats which had received a fat-free diet. The curative effect of linoleic acid has been repeatedly confirmed [Evans & Lepkovsky, 1932; Tange, 1932; Becker, 1934; Becker, 1935; Moore, 1937; Turpeinen, 1938]. As regards linolenic acid the facts are not so well established. The experiments with pure methyl linolenate seem to have been repeated only by Tange [1932], who concluded that linoleic and linolenic acids were equally effective in promoting weight increase and bringing about a cure. His experiments do not, however, appear entirely to justify this conclusion. He describes experiments in which groups of 2 and 3 rats were dosed daily with one drop of methyl linoleate and linolenate respectively, and says that when growth was somewhat retarded the dose of methyl linolenate was increased to 2 drops but no great improvement was observed; he further reports that this retardation and irregularity, which were not shown with linoleic acid, seemed to be rather influenced by heat. A chart shows as equal the growth curves of two rats dosed with methyl linoleate and of two dosed with methyl linolenate, but no details are given as to the size of the doses.

Since linolenic acid is always accompanied in plant oils by linoleic acid,

it is essential that the linolenic hexabromide from which the linolenic acid is prepared should be carefully purified. In the experiments of Burr & Burr the samples of linoleic and linolenic acids used were obtained from maize and linseed oils respectively. The unsaturated acids were brominated and the resulting tetra- and hexa-bromostearic acids were carefully purified by recrystallization. These were then debrominated, esterified and distilled at less than 1 mm. pressure. The preparation of linolenic hexabromide melted at 180 to 181°, so that it could not have contained any appreciable quantity of the linoleic tetrabromide. Burr & Burr tested the material thus prepared on a group of 3 rats, each receiving 3 drops of methyl linolenate daily, and on a group of 2 rats, each receiving 3 drops of methyl linoleate daily. The average total weight increases of the two groups in 63 days were almost identical, being respectively 31 and 31·5 g.

Chemical preparation. In the present experiments two preparations of linolenic hexabromide, derived from linseed oil and melting at 180–182° and at 182·5° respectively, were used for the preparation of methyl linolenate; the Br contents were 62·0 and 63·06 % for the two samples, the theoretical value for linolenic hexabromide being 63·3 %. The iodine values of the two specimens of methyl linolenate used in the feeding tests were 230·6 and 245 respectively.

The linoleic acid prepared from maize oil was converted into the linoleic tetrabromide, the M.P. of which was 115°; the Br contents were 54·4 and 53·7 %, the theoretical value being 53·3 %. This material was debrominated and esterified, and the methyl linoleate thus prepared gave iodine values of 165·6 and 166·5. To avoid oxidation the unsaturated esters were sealed in small tubes filled with nitrogen and kept at 0°. When a tube was in use for feeding the I.V. was tested at intervals to see that no marked oxidation had taken place. Under these conditions the I.V. of the methyl linoleate had fallen after 24 days from 166·5 to 163·1 and that of the methyl linolenate from 245 to 240.

A sample of methyl β-linoleate was prepared by debrominating the liquid form of linoleic tetrabromide. It had an iodine value of 135·2.

The sample of linseed oil used for comparison in the feeding tests had I.V. 180 and contained about 26 % linoleic and about 44 % linolenic acid.

Biological tests. The biological tests were essentially a comparison between the potencies of methyl linoleate and methyl linolenate. Groups of 7–9 rats received a daily dose of 6 drops (0·08 g.) of one of these two substances or served as negative controls with no addition (see Tables I a and b). The average total weight increase in 35 days for the group receiving methyl linoleate was 23 g., for that receiving methyl linolenate 8 g. and for the negative control group 2 g. As regards the skin lesions of the ankles, all but one of the rats receiving methyl linoleate showed complete healing in 35 days; of those receiving methyl linolenate all showed partial, but none complete, healing in the period, and the negative control group showed no healing at all.

In addition two animals received daily 5 drops (about 0·08 g.) of linseed oil, one received 6 drops (0·08 g.) of methyl β-linoleate and four received 1 drop (0·013 g.) of methyl linoleate. The numbers of rats for these tests are small but reference to Tables I a and I b shows that the average behaviour of these groups both as regards cure of lesions and resumption of weight increase was intermediate between that of the group receiving 0·08 g. methyl linoleate, and that receiving 0·08 g. methyl linolenate. The average weight increase of 14 g. shown by the rats receiving one drop of methyl linoleate was greater than that of 8 g. shown by the rats receiving 6 drops of methyl linolenate.

The behaviour of one rat was of particular interest. After having received 6 drops methyl linolenate daily for 13 days it had lost 19 g. in weight and was in

Table I a. *Total wt. increase in 35 days of young rats which had received a diet devoid of fat for about 120 days, and then were given methyl linoleate, methyl β-linoleate, methyl linolenate, no supplement or linseed oil*

(Results marked with the same symbol *, † etc., apply to the same individual rat in two different tests.)

Litter no.	Sex	Methyl linoleate 0·013 g. daily (g.)	Methyl linoleate 0·08 g. daily (g.)	Methyl β-linoleate 0·08 g. daily (g.)	Methyl linolenate 0·08 g. daily (g.)	No supplement negative controls (g.)	Linseed oil 0·08 g. daily (g.)
4330	♂	17*	13	.	.	- 10*	.
	♀	.	.	.	14†	- 1	.
	♀	16	.	.	7†	.	.
4329	♂	.	19	.	2	- 17	20
	♀	.	30	.	9	2‡	20
	♀	12‡	.	.	.	.	.
4338	♀	.	31	16	8	5§	.
	♀	.	18	.	- 19·‖ (13 days)	.	.
	♀	13§	39′	.	25	.	.
4350	♂	.	.	.	3	3	.
	♀	.	10	.	3	5	.
Av.		14	23	16	8	2	20

Table 1b. *Healing of skin symptoms in 35 days. Same series as above. Three degrees of healing recognized: complete + +, partial +, none θ*

Litter no.	Sex	Methyl linoleate 0·013 g. daily	Methyl linoleate 0·08 g. daily	Methyl β-linoleate 0·08 g. daily	Methyl linolenate 0·08 g. daily	No supplement negative controls	Linseed oil 0·08 g. daily
4330	♂	+ +*	+ +	.	.	θ*	.
	♀	.	.	.	+†	θ	.
	♀	+	.	.	+†	.	.
4329	♂	.	+ +	.	+	θ	+
	♀	.	+ +	.	+‡	θ	+
	♀	+‡	.	.	.	.	.
4338	♀	.	+ +	⊤	+	θ§	.
	♀	.	+ +	.	θ‖ (13 days)	.	.
	♀	+§	+‖	.	+	.	.
4350	♂	.	.	.	+	θ	.
	♀	.	+ +	.	+	θ	.

a very precarious condition. The dose was changed to 6 drops daily of methyl linoleate; the rat at once ceased to lose weight and quickly began to gain, making a total weight increase of 39 g. in the 35 days immediately ensuing (see Table I a, b, rat 4338 ♀‖). The skin lesions of the ankles were not, however, completely cured within this period. Rats did not usually lose weight when receiving methyl linolenate, so that this is the only case in which so marked a curative action of methyl linoleate in contrast with methyl linolenate was demonstrated.

A certain number of the rats continued to receive the unsupplemented deficient diet after the end of the 35-day period of dosage with methyl linoleate or linolenate, and they were observed for irregular periods varying from 30 to 80 days. Their behaviour showed a powerful and sustained action of methyl

linoleate and a weak and transitory one of methyl linolenate. Of 5 rats receiving the former, all continued to gain in weight slowly and the unhealed lesions of skin and tail, excluding the ankles which were already healed, progressed steadily towards cure. Of 3 rats which received 6 drops of methyl linolenate, only one made any sustained weight increase and none showed any progress of the cure; the ankles, which were only partly healed at the end of the period of dosage, never showed any tendency to complete healing (see Table II).

Table II. *Subsequent behaviour of rats receiving the unsupplemented fat-free diet in the period immediately following a 35-day period of dosage with 0·08 g. daily of methyl linoleate or methyl linolenate*

Litter no.	Sex	Previous daily supplement	Length of period without supplement days	Total wt. increase in period without supplement g.	Behaviour of symptoms in period without supplement
4330	♂	Methyl linoleate 0·08 g.	80	33	All skin and tail lesions continuing to progress towards normal
4329	♂	,,	70	28	
4329	♀	,,	70	20	
4338	♀	,,	30	16	
4338	♀	,,	36	13	
4330	♀	Methyl linolenate 0·08 g.	45	0 { +7 −7 }	No progress in healing of lesions. Partly healed ankles showing no improvement
4329	♀	,,	42	1 { +4 −3 }	
4329	♂	,,	45	12	

All the experimental evidence thus obtained, therefore, went to indicate that methyl linoleate exercised a potent and prolonged action in counteracting the effects of a fat-free diet, while methyl linolenate had only a weak and transitory action.

Discussion. The difference thus established is of especial interest since there is general agreement that whilst linoleic acid is normally a constituent of the body lipins, linolenic acid is only present in small amounts, as the result of administering it in exceptionally large amounts [Ellis & Isbell, 1926; Spadola & Ellis, 1936]. Thus Hartley [1909] found evidence of the presence in the liver, heart and kidney of pigs of unsaturated acids with one, two, and four double bonds. Snider & Bloor [1932–3] found oleic, linoleic and arachidonic acids in ox liver lecithin, and failed to find linolenic acid, a result confirmed by Klenk & Schoenebeck [1932]. Turner [1930] found no linolenic acid in sheep's liver. Eckstein [1929] found linoleic and arachidonic acids in rat tissues but no evidence of linolenic acid. Levene & Rolf [1926] described linolenic acid as exceeding linoleic acid in amount in liver lecithin, but the hexabromide described, being soluble in ether, does not agree in properties with linolenic hexabromide. It seems certain, therefore, that though linolenic acid may be consumed in large quantities it is not taken up as a constituent of the body lecithins. Since it is not stored in the body it is not surprising that after its administration has been stopped, no further increase in weight or improvement of symptoms takes place in the rat. Linolenic acid must be more rapidly transformed on entering the body than in linoleic acid, and it is possibly owing to this change that methyl linolenate is so markedly inferior to methyl linoleate in its effect in counteracting the effects of a fat-free diet.

Effects of elaidized linoleic and linolenic acids. Tange [1932] added 0·5 % elaidic acid to the fat-free diet of 5 rats of which two failed to show weight increase.

He reports that this acid was ineffective in curing symptoms and behaved like oleic acid.

Preparation. Linoleic and linolenic acids were elaidized according to the directions of Griffiths & Hilditch [1932] and the methyl esters were used for the biological test. The esters were orange in colour; the I.V. of the original methyl α-linoleate was 165·6, and after elaidization it was 157·2. The I.V. of the original methyl α-linolenate was 230·6, and after elaidization it was 215.

Biological test. One rat received 0·08 g. daily of elaidized methyl α-linoleate and showed a weight increase of 24 g. in the experimental period, with partial healing of skin lesions. Another rat received the same amount of elaidized methyl α-linolenate and showed no weight increase but about the same degree of healing; it was found to be suffering from the chronic lung affection of rats which would affect adversely its capacity to put on weight (see Table III). The elaidized methyl α-linoleate still contained a large proportion of non-elaidized linoleate; the degree of healing was inferior to that previously observed with the same dose of methyl linoleate.

(2) *Examination of the potencies of certain oxidation products of linoleic and linolenic acids. (a) Tetrahydroxystearic acids: tests on mixtures of α- and β-, and of γ- and δ-tetrahydroxystearic acids. (b) Dioxidostearic acid. (c) Hexa-hydroxystearic acids: linusic and isolinusic acids.*

(a) *Tetrahydroxystearic acids.* Nothing is known as to the changes which linoleic and linolenic acids undergo in the body. It seemed possible that, as a further stage in metabolism, oxidation products might be formed. This possibility cannot be tested exhaustively even for the hydroxystearic acids, since few of them have been isolated. Thus eight racemic forms of tetrahydroxystearic acid may theoretically be derived from linoleic acid but only four of these are known; α- and β-tetrahydroxy-stearic acids, melting respectively at 154·7° and 171·3°, have been obtained by the action of $KMnO_4$ on linoleic acid in alkaline solution. The γ- and δ-forms were obtained by Nicolet & Cox [1922] by the addition of HOBr to linoleic acid and the conversion of the hydroxybromo-derivatives into the tetrahydroxy-acids. The γ-form melted at 144·5°, the δ-form at 135°.

Preparation. Mixtures of the 9:10:12:13-α- and β-tetrahydroxy-acids were prepared with M.P. 164–166°, and of the 9:10:12:13-γ- and δ-tetrahydroxy-acids with M.P. 145–146° by the above methods. These two mixtures were used for biological tests.

Biological tests. Three rats received 0·2 g. daily of the mixture of the α- and β-forms, and one rat received 0·2 g. of the mixture of γ- and δ-forms (see Table III), but no weight increase or amelioration of skin symptoms occurred in any case. Thus no indication of the activity possessed by linoleic acid was shown by these tetrahydroxy-addition products of it.

(b) *Dioxidostearic acid. Preparation.* Dioxidostearic acid was prepared by the method of Green & Hilditch [1935], by the oxidation of methyl linoleate with perbenzoic acid in chloroform solution and recrystallization from hot 70 % alcohol. It melted at 78–79°.

Biological test. A daily dose of 0·2 g. of the preparation was added to the diet of one rat for 35 days. It showed no gain in weight and no healing of skin symptoms; a litter-mate negative control gained 7 g. in the same period (see Table III).

Table III. *Total wt. increase and healing of skin symptoms in young rats which had received a diet devoid of fat for about 120 days and then various supplementary materials for differing periods*

Litter no.	Sex	Material	Dose daily g.	Duration of dosing days	Wt. increase g.	Healing of symptoms
4329	♂	Elaidized methyl α-linoleate	0·08	35	24	+
4329	♀	Elaidized methyl α-linolenate	0·08	35	0 (pneumonia)	+
4075	♀	9:10:12:13-α- and β-tetrahydroxy-stearic acid	0·2	35	0	θ
4076	♀	,, ,,	0·2	35	0	θ
4330	♂	,, ,,	0·2	35	- 9	θ
4330	♂	9:10:12:13-γ- and δ-tetrahydroxy-stearic acid	0·2	35	- 9	θ
4338	♀	Dioxidostearic acid	0·2	35	- 3	θ
4350	♂	Methyl docosahexaenoate	0·06–0·1	35	24	? < +
4350	♀	,,	0·06–0·1	35	21	? < +
4273	♂	Chaulmoogra oil	0·16	13	- 5	θ
		Chaulmoogric acid	0·2	5	0	θ
4330	♂	Methyl arachidate	0·3	15	9	θ
4273	♀	9:10:12-trihydroxystearic acid	0·2	35	4	θ

(c) *Hexahydroxystearic acids*: *linusic and* isolinusic *acids*. Thirty-two racemic isomerides of the hexahydroxystearic acids derived from linolenic acid are theoretically possible; the racemic linusic and *isolinusic* acids obtained by oxidation with $KMnO_4$ of the unsaturated acids of linseed oil [Rollett, 1909] are the only ones known.

Preparation. Linusic acid, M.P. 196–197° and *isolinusic* acid, M.P. 172–174°, were prepared as above.

Biological tests. The results are set out in Tables IV a, b. Five rats received 0·2 g. daily of linusic acid; 7 received the same amount of *isolinusic* acid, and 2 received 0·1 to 0·13 g. daily of a mixture of both acids. All tended to show some weight increase, the 12 animals which received 0·2 g. daily giving an average total weight increase for the experimental period of 6 g., as compared with an average total weight increase of 3 g. for 7 litter-mate negative controls with no supplement, and of 20 g. for 5 litter-mate positive controls receiving 3–10 drops (0·05–0·16 g.) linseed oil daily. This performance appears to represent a definite though small weight increase by the rats receiving the hexahydroxystearic acids, not greatly inferior to that of rats receiving methyl linolenate, as already described (see Table I a). As regards the healing of skin lesions the rats receiving the hexahydroxystearic acids behaved like the negative controls. It is regrettable that rats receiving methyl linolenate were not included simultaneously in this comparison; when it was begun the inferiority of linolenic to linoleic acid had not been established and it was thought that the mixture of both these acids, represented by linseed oil, would be a satisfactory material for a positive control.

The faeces of the rats fed with the tetrahydroxy-acids in the previous experiment and the hexahydroxy-acids in the present experiment were large and pale in colour and obviously contained a large amount of the hydroxy-acids administered. Indeed it is doubtful how much of substances of such high melting points and so insoluble in water could be absorbed; the amounts absorbed can only have been a small fraction of the dose administered. The solubility in water of the hexahydroxy-acids is appreciably greater than that of the tetrahydroxy-acids, and possibly more of these may pass through the intestinal wall. For a part of the experimental period one rat received ethyl linusate and another ethyl

Table IV a. *Total wt. increase in 35 days of young rats which had received a diet devoid of fat for about 120 days, and then were given linusic acid, isolinusic acid, no supplement or linseed oil*

Litter no.	Sex	Linusic acid 0·2 g. daily g.	isoLinusic acid 0·2 g. daily g.	No supplement g.	Linseed oil 3–10 drops daily (0·05 to 0·16 g.) g.
4075	♀	9	9	.	17
	♂	.	.	8	.
4076	♀	11	12	4	17
4273	♀	− 4*	− 1*	− 1	.
	♀	0*	.	.	.
	♂	.	−13	− 13	25
4330	♀	.	4	− 1	.
4350	♀	6	8	8	10
3262	♀	.	7	4	25
		Linusic and isolinusic acids mixed 0·1–0·13 g.			
2907	♀	9		− 1	.
2931	♂	35†		6†	26†
Av.		5	6	3	20
		8			

* Experiment lasted 30 instead of 35 days.
† Experiment started on 75th instead of about 120th day.

Table IV b. *Healing of skin symptoms in 35 days. Same series as in Table IV a. Three degrees of healing recognized, complete + +, partial +, and none θ*

Litter no.	Sex	Linusic acid 0·2 g. daily	isoLinusic acid 0·2 g. daily	No supplement	Linseed oil 3–10 drops daily (0·05–0·16 g.)
4075	♀	θ	θ	.	+ +
	♂	.	.	θ	.
4076	♀	θ	θ	θ	+
4273	♀	θ*	θ*	θ	.
	♀	θ*	.	.	.
	♂	.	θ	θ	+
4330	♀	.	θ	θ	.
4350	♀	θ	θ	θ	+ +
3262	♀	.	θ	θ	+
		Linusic and isolinusic acids mixed 0·1–0·13 g.			
2907	♀	θ		θ	.
2931	♂	θ†		θ†	+ + †

* Experiment lasted 30 instead of 35 days.
† Experiment started on 75th instead of about 120th day.

isolinusate, instead of the corresponding acids, in the hope that the esters might be better absorbed, but weight increase was not favourably affected. An attempt to promote absorption was also made by adding about 0·25 g. daily of coconut oil simultaneously with the dose of 0·2 g. daily of linusic acid to the diet of one rat for a part of the experimental period, but without result.

The experiments with these hydroxy-derivatives cannot, therefore, be regarded as conclusive since there was no test of the amount absorbed. The small positive effect on weight increase observed in the case of linusic and *iso*linusic acid seems, however, even more convincing when it is remembered that a considerable proportion of the original dose was certainly excreted in the faeces.

(3) *Comparison of the potency of the unsaturated acids from lard and from linseed oil*

When Burr & Burr [1929] first described the deficiency disease produced by a fat-free diet they found that the condition was cured or prevented by the inclusion of 2 % lard in the diet. They referred to lard as one of the best curative fats and suggested that this may possibly be due to the presence of arachidonic acid in the lard fat. Later Burr *et al.* [1932] tested the efficacy of arachidonic acid by replacing 10 % of a mixture of the esters of linoleic and linolenic acids by methyl arachidonate and comparing the effect of 3 drops of the mixture with that of 3 drops of the original ester mixture. The mixture containing the arachidonate proved to be appreciably less active in promoting weight increase than the original mixture. Turpeinen [1938], on the other hand, estimated the effect on weight increase of pure methyl arachidonate and found it three times as potent as methyl linoleate. He does not, however, give any account of its healing effect on the skin lesions, though he found it potent to restore the oestrous cycle towards normal.

The amount of arachidonic acid in lard is usually less than 0·1 % [cf. Ellis & Isbell, 1926; Spadola & Ellis, 1936]; the sample used by Burr & Burr contained 0·06 % so that it does not seem probable that the potency of the lard depended to any great extent on its content of arachidonic acid. Its effect must, therefore, have been due chiefly to its content of linoleic acid. In the lard derived from maize-fed pigs used by Burr & Burr, the percentage of linoleic acid was 6·7. The percentage of linoleic acid in lard is extremely variable, being dependent on the previous diet of the pig. The lard of an animal fed with a ration containing oils rich in linoleic acid may contain as much as 20 % of linoleic acid, and when soya bean has been included in the food 35 to 36 % has been found [Banks & Hilditch, 1932; Ellis & Isbell, 1926; Ellis *et al.* 1931], but usually the linoleic acid content is below 10 %. Percentages varying from 23 to 70 are given for the linoleic, and from 18 to 49 for the linolenic, acid content of linseed oil, but since the proportions of the two acids vary inversely to one another, the iodine value of the oil is fairly constant. Burr & Burr [1929] and Burr *et al.* [1932] found lard less potent than linseed oil.

Preparation. The methyl esters of the unsaturated acid fraction were prepared from American lard, distilled *in vacuo*, and a fraction with i.v. 111·7 was used for the biological tests. This would correspond with a mixture of about 70 % oleic and about 30 % linoleic acids.

A fraction of the unsaturated acids from linseed oil was also prepared; it had i.v. 199 to 200 and contained approximately 20 % oleic, 30 % linoleic and 50 % linolenic acid. The linoleic acid content of the fraction derived from the lard was thus about the same as that of the fraction derived from the linseed oil, but the linseed oil fraction contained in addition nearly double the amount of linolenic acid.

Biological tests. Groups of 4 litter-mate rats received 3 to 5 drops (about 0·05–0·08 g.) daily of the fraction of unsaturated acids from lard and from linseed oils, two animals in each group receiving 3 and two 5 drops. The average

total weight increase was 29 g. in both groups, and all the animals showed partial healing of the ankles to about the same extent (see Table V). It is therefore concluded that the activities of these two fractions were about the same.

Table V. *Total wt. increase and healing of skin symptoms in 35 days of young rats which had received a diet devoid of fat for about 120 days, and were then given unsaturated fatty acid fractions from lard or linseed oil*

Litter no.	Sex	Linseed oil acids 3–5 drops (0·05–0·08 g.) g.	Lard acids 3–5 drops (0·05–0·08 g.) g.	Linseed oil acids 3–5 drops (0·05–0·08 g.)	Lard acids 3–5 drops (0·05–0·08 g.)
3262	♂	36	27	+	+
	♀	21	21	+	+
1255's	♂	40	48	+	+
1258's	♀	16	22	+	+
Av.		28	29	.	.

These experiments seem to afford additional confirmation of the great superiority of linoleic over linolenic acid as a curative agent, for the two fractions contained about the same amount of linoleic acid, but the linseed oil contained a large amount of linolenic acid in addition.

Lard and linseed oil vary so much in their linoleic acid contents that considerable discrepancies between the results of different workers must be expected, and feeding experiments in which different samples of lard are used can be justly compared only when the linoleic acid contents have first been determined.

(4) *Comparison of the potencies of raisin seed oil and of linseed oil*

Raisin seed oil, which has only comparatively recently appeared on the market, is a by-product of the wine industry. A sample of material of Californian origin was contributed by Messrs W. J. Bush and Co., Ltd. for a biological test. This sample of raisin seed oil proved to have I.V. 122·4. It contained 93·5 % of fatty acids, the I.V. of which was 126·7, with 1·07 % of unsaponifiable matter. On bromination no ether insoluble bromide was obtained, and the percentage of linoleic acid was therefore calculated to be 38·6, no evidence of the presence of linolenic acid having been obtained.

The linseed oil used was that already described (p. 2166) as having a content of about 26 % linoleic, and about 44 % linolenic, acid.

Biological test. Four litter-mate rats received for 35 days 5 drops (about 0·08 g.) daily of the raisin seed oil, four received the same amount of linseed oil, and

Table VI. *Total wt. increase and healing of skin symptoms in 35 days of young rats which had received a diet devoid of fat for about 120 days, and then were given linseed oil or raisin seed oil or no supplement*

Litter no.	Sex	Linseed oil 5 drops (0·08 g.) g.	Raisin seed oil 5 drops (0·08 g.) g.	No supplement negative controls g.	Linseed oil 5 drops (0·08 g.)	Raisin seed oil 5 drops (0·08 g.)	No supplement negative controls
4075	♂	39	52	10	+	+ +	θ
	♂	.	31	.	.	+ +	.
	♀	16	18	.	+ +	+ +	.
4076	♂	28	.	0	+	.	θ
	♀	17	12	5	+	+ +	θ
Av.		25	28	5			

three received no supplement. The average total weight increases in the period were 28, 25 and 5 g., respectively. All the four rats receiving raisin seed oil, and one rat receiving linseed oil, showed complete healing of the ankles in the period; the other three rats receiving linseed oil showed partial healing; the three receiving no supplement showed no healing (see Table VI).

A slight superiority of raisin seed oil over linseed oil seems therefore to be established. The result harmonizes well with the other results obtained and confirms the marked inferiority of linolenic to linoleic acid, since the linseed oil sample had a large content of linolenic acid, but somewhat less linoleic acid than the raisin seed oil.

(5) *Test of the potency of the methyl ester of the docosahexaenoic acid isolated from cod liver oil by Farmer & Van den Heuvel*

There is a mass of evidence in the literature showing that cod liver oil, although ineffective in curing the skin lesions produced by a fat-free diet, is effective in promoting weight increase. Thus Sinclair [1929–30] found that this oil, added as 10% of the otherwise fat-free diet of rats, did not cure scaliness, whereas 1% of lard was effective. Graham & Griffith [1930–31] reported a daily dose of 9–12 drops of cod liver oil as ineffective while wheat germ oil and lard cured well. Burr *et al.* [1930–31] gave 2–5 drops of cod liver oil daily and found weight increase renewed though the scaly tail was not cured. McAmis *et al.* [1929] found the rate of weight increase of fat-deprived rats much improved when 0·02 g. cod liver oil was added to the daily ration. These results would indicate the absence of linoleic acid from cod liver oil and the presence of other unsaturated acids able to promote weight increase but unable to alleviate the skin lesions [Burr *et al.*, 1930–31]. From the figures given by Banks *et al.* [1933] for the iodine values of the unsaturated acids derived from the depot fat of rats which had received a fat-free diet supplemented with cod liver oil, it is clear that when cod liver oil was given, highly unsaturated acids were laid down in the depot fat.

The composition of cod liver oil was investigated by Guha *et al.* [1930] who examined the methyl ester fractions of the unsaturated acids obtained by distillation under a pressure of 1 mm. If it is assumed that the iodine value of the fraction containing 18 carbon atoms is entirely due to the presence of oleic and linoleic acids, the proportion of linoleic acid would correspond with a content of about 12% in the original oil. The fraction containing 18 carbon atoms formed 18–32% of the total acids and absorbed from 2·8 to 4 atomic proportions of hydrogen. There was, however, no actual identification of linoleic or linolenic acid, and the same values could be obtained from mixtures of isomeric acids. In view of the absence of healing of the skin lesions when large doses of cod liver oil are added to the fat-free diet, it is difficult to believe that any appreciable amount of linoleic acid can be present in the oil [cf. Burr *et al.*, 1930–31].

Farmer & Van den Heuvel [1938, 2] have recently pointed out that, in all fractionations of the methyl esters of cod liver oil previously carried out, changes occurred which pointed to the occurrence of alterations in structure during distillation. After distilling the esters, even at a pressure of 0·1 mm., evidence of the presence of conjugated double bonds was obtained whereas there was no evidence of their presence before distillation. Much polymerization also occurred and the fractions isolated consisted of constant boiling mixtures. Farmer & Van den Heuvel [1938, 1] have, therefore, used the method of molecular distillation in which pressure was reduced to 0·001 mm. In this way, from 950 g. unsaturated acids, 225 g. of an acid containing 22 carbon atoms and 6 double bonds was isolated, no conjugated double bonds being present in the molecule

[Farmer & Van den Heuvel, 1938, 2]. The fraction containing 18 carbon atoms, when hydrogenated, gave pure stearic acid; the value for the hydrogenation number indicated that it contained 2·7 double bonds, so that in composition it approximated more nearly to linolenic than to linoleic acid.

A sample of the methyl ester of the C_{22} hexaenoic acid was contributed by Dr Farmer and was used for a biological test.

Biological test. The methyl ester of the docosahexaenoic acid had a strong fishy odour and was much disliked by the rats. It also showed in a high degree the property already mentioned (see p. 2164) of destroying the upper layers of the skin about the mouth, wherever it was allowed to come into contact with them, though no damage appeared to be caused to the mucous membrane of the mouth or intestinal tract. By intimate admixture with the daily yeast dose its consumption was satisfactorily secured.

Two rats received a daily dose of 5–8 drops (0·06–0·1 g.) of the material for 35 days, in which period the weight increase was 21 and 24 g. Such weight increase much improved the appearance of the animals, making it difficult to decide whether there was or was not some small improvement in the skin lesions; the ankles were, however, still rough and quite unhealed at the end of the period (see Table III). After the 5 weeks' experimental period the two animals were maintained on the same diet, without addition of the daily dose of acid, for another 14 days, on the chance that cure of the skin symptoms might continue and become more convincing as had been found to be the case with linoleic acid, but this did not occur. One rat, however, added another 5 g. of weight and the other 12 g. in this period. The behaviour of the rats was therefore in marked contrast with that of rats receiving linoleic acid, and there seem to be adequate grounds for distinguishing between the effects of the highly unsaturated acids of cod liver oil, which enable body fat to be laid down, and those of linoleic acid, which in addition to promoting weight increase, produce rapid amelioration and final cure of skin lesions.

(6) *Tests for potencies of chaulmoogra oil and chaulmoogric acid, methyl arachidate and 9:10:12-trihydroxystearic acid*

Chaulmoogra oil. The sample of oil used had I.V. 101·3. One rat received 10 drops (about 0·16 g.) daily for 13 days, but it lost weight and the oil appeared to be actually toxic (see Table III).

Chaulmoogric acid was prepared from chaulmoogra oil. It had M.P. 65·5° and I.V. 91·1. The same rat as above received 0·2 g. daily for 5 days; the material was badly consumed but appeared quite inactive, a result in agreement with that of Turpeinen [1938].

Methyl arachidate. In view of Turpeinen's work on the activity of arachidonic acid, it was of interest to see whether any evidence of activity could be obtained which would indicate the unsaturation within the body of arachidic to arachidonic acid. From arachis oil a fraction of saturated acid M.P. 72–75° was prepared, consisting mainly of arachidic acid. This was converted into the methyl ester. Owing to the high melting point the material would probably be badly absorbed in the biological test.

Biological test. One rat received 0·3 g. daily of the material for 15 days during which period it lost 9 g. and showed no alleviation of symptoms. The result was regarded as completely negative (see Table III).

9:10:12-Trihydroxystearic acid. The acid was prepared by oxidizing ricinoleic acid with $KMnO_4$; crystallized from alcohol it melted at 131–134°.

Biological test. One rat received 0·2 g. daily of the material for 35 days; it lost 4 g. and showed no improvement in skin symptoms. Like the ricinoleic acid tested by Turpeinen, it was thus completely inactive (see Table III).

SUMMARY

1. The principal symptoms of the deficiency disease described by Burr & Burr as affecting rats maintained on a complete diet devoid only of fat were reproduced.

2. The efficacy of various materials adequately to supplement the fat-free diet was studied. Resumption of weight increase and healing of skin symptoms were the criteria used.

3. A rough method of estimating quantitatively the rate of healing of skin lesions is described. The importance of using some additional criterion more specific than weight increase is stressed.

4. The efficacies of methyl linoleate and methyl linolenate were compared: methyl linoleate was found much more potent and lasting in action than methyl linolenate, which possessed perhaps no more than one-sixth of the potency of methyl linoleate.

5. Various oxidation products of linoleic and linolenic acid were prepared and tested. Mixtures of α- and β- and of γ- and δ-tetrahydroxystearic acids in daily doses of 0·2 g. were completely ineffective in promoting weight increase or curing skin lesions in rats. Dioxidostearic acid was similarly inactive.

The hexahydroxy-derivatives, linusic and *iso*linusic acids, given in daily doses of 0·2 g., failed to benefit skin lesions but promoted a small but definite weight increase nearly equal to that evoked by administration of methyl linolenate in a daily dose of 0·08 g. All these oxidation products were solid substances of high melting point and were certainly absorbed only to a very limited extent by the rat's intestine.

6. The potency of a fraction of the unsaturated acids from lard was compared with that of a similar fraction from linseed oil and was found to be about the same, both as regards promotion of weight increase and healing of skin lesions.

7. A similar comparison was made of linseed oil with raisin seed oil: the latter was found slightly the more potent by both criteria. The linoleic acid contents of these two materials and of the fractions of linseed oil and lard were not greatly dissimilar but the linseed oil and its acids both contained 40 % to 50 % linolenic acid in addition. These results therefore confirm the superior potency of linoleic to linolenic acid.

8. The methyl ester of the docosahexaenoic acid isolated by Farmer and Van den Heuvel from cod liver oil was tested and found to be potent in promoting weight increase but to have little or no action in curing skin lesions.

9. Chaulmoogra oil and chaulmoogric acid, methyl arachidate and 9:10:12-trihydroxystearic acid were also tested and found inactive.

10. It is concluded that the ability of unsaturated fatty acids to supplement a fat-free diet in promoting weight increase is not necessarily associated with ability to heal skin lesions.

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CCLXXXII. THE NATURE OF THE FATTY ACIDS STORED BY THE LIVER IN THE FAT-DEFICIENCY DISEASE OF RATS

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THE part played by those unsaturated fatty acids which have been shown to be essential for the maintenance of rats in normal health is as yet quite unknown. A first step in elucidating this problem was to gain further knowledge as to the nature of the fatty acids stored when the animals were kept on a fat-deficient diet, and to observe the changes which occurred in them when the curative acids were fed.

At the conclusion of feeding experiments carried out in co-operation with Hume *et al.* [1938], we selected five groups of rats and examined the fatty acids present in the livers. The long period of preparation necessary before symptoms are sufficiently well established for curative measures to be tested made strict economy desirable and in most cases before the rats were killed the negative controls had been utilized for further tests. It was not, therefore, possible to obtain a large group of negative controls for the isolation of the liver fatty acids and we had not sufficient material to make a separation into phospholipin and neutral fat. In normal animals the greater part of the liver fatty acids comes from the phospholipins; in the ox, Bloor & Snider [1930] give the proportion of phospholipin fatty acid to acid from neutral fat as 6 : 1; this figure is in close agreement with those given by Klenk & Schoenebeck [1932]. Theis [1928], on the other hand, gives the ratio as 5 : 4. Monaghan [1932] found that in fasting animals the proportion of neutral fat was much raised. It seems probable that most of the highly unsaturated fatty acids came from the phospholipin fraction, but definite evidence on this point is still required.

The five groups of rats selected were:

(1) *Two rats* ($\male + \female$) *which served as our negative controls.* These weighed 42 and 45 g. respectively when placed on the fat-free diet. The diet was continued for 18 weeks and after the growth curve had remained flat for 6 weeks they received respectively daily doses of 0·2 g. linusic and *iso*linusic acids. These doses were continued for 5 weeks, during which period the weight of the linusic-fed rat increased by 5 g. and that of the one fed with *iso*linusic by 8 g. For the next 7½ weeks before being killed they again received the fat-deficient diet; these were chosen as being the nearest to completely negative controls available at the end of the feeding experiments.

Although the linusic-fed rat only gained 5 g. in weight during the 5 weeks period of dosing, 17½ g. increase was recorded in the 3½ weeks immediately following cessation of the dose, then the weight remained stationary until the rat was killed 3½ weeks later. Since both rats had received the fat-free diet for 7 weeks before they were killed and prior to that the comparatively inactive hexahydroxy-acids had been fed, these rats may probably be regarded as characteristic examples of rats fed on a fat-free diet.

(2) *Rats receiving methyl linoleate.* Three rats (♀) weighing 44, 45 and 46 g. respectively when transferred to the fat-deficient diet, were utilized. One of these, after it had been on this diet for 20 weeks, was given a daily dose of 6 drops methyl linoleate for a period of 6 weeks during which its weight rose from 121 to 152 g. A month's cessation of dosing followed during which the rat's weight increased by 16 g. and finally, for its last 3 weeks, it received 1 drop linoleate daily. The other two rats, after receiving the fat-deficient diet for 6 months, were both dosed with 1 drop linoleate daily for 38 days, the respective gains in weight being 12 and 15 g. This dose was shown to produce amelioration but not cure of symptoms and the rats, therefore, probably do not show the typical results of rats fed with the optimum linoleate dose.

(3) *Rats receiving methyl linolenate.* Two rats (♀) which had received the fat-free diet for 16 weeks were given, respectively, during the course of 1 month, a daily dose of 0·2 g. dioxidostearic acid and 1 drop of methyl linolenate. Finally for 38 days before they were killed, each received 6 drops linolenate daily. The effects produced may therefore be considered as characteristic for rats fed with methyl linolenate.

(4) *Rats receiving linseed oil.* Six rats (3 ♂, 3 ♀) had received the fat-free diet for from 8 to 10 months. After 4 months one had been given 0·2 g. linusic acid daily for 5 weeks and then after a month's interval a daily dose of 0·2 g. trihydroxystearic acid for another period of 5 weeks. In all cases for 2 to 3 months before being killed, each rat had received a supplement of 5 to 15 drops linseed oil daily. The typical fatty acids stored by rats on a diet containing linseed oil might, therefore, be expected.

(5) *Rats fed with docosahexaenoic acid methyl ester (from cod liver oil).* Two rats were utilized (1 ♂, 1 ♀) which had served as negative controls for 6 months. During 2 weeks they each received a daily dose of 8 drops of the above ester [Farmer & Van den Heuvel, 1938] and for the subsequent 3 weeks, 5 drops daily. Dosage had ceased a fortnight before the rats were killed. Possibly if the rats had been killed whilst the diet still contained the ester supplement, the i.v. of the liver fatty acids might have been higher.

As soon as the animals were killed, the livers were removed, weighed and immediately thrown into a mixture of equal parts of alcohol, water and caustic potash heated on a water bath. After it had been extracted with ether, the soap solution was acidified and the fatty acids were extracted by light petroleum.

Table I

Supplement	No. rats	Total wt. livers g.	Av. wt. liver per rat g.	Total wt. fatty acids g.	Wt. acids in 100 g. liver g.	(a) unsaturated	(b) saturated	i.v. unsaturated acids
						% acids		
Group I. None	2 (♂ + ♀)	16·5	8·2	0·62	3·7	62·1	37·9	121·2
Group II. Methyl linoleate	3 (♀)	21·8	7·3	0·57	2·6	62·1	37·9	163·6
Group III. Methyl linolenate	2 (♀)	15·0	7·5	0·36	2·4	62·3	37·7	215·0
Group IV. Linseed oil	6 (3 ♂ + 3 ♀)	55·0	9·2	1·25	2·3	61·7	38·3	202·8
Group V. Methyl ester of cod liver oil acid (docosahexaenoic acid)	2 (♂ + ♀)	17·3	8·7	0·50	2·9	61·6	38·4	162·3

Three interesting facts emerge from the results recorded in Table I:

(1) The weight of fatty acid calculated per 100 g. liver tissue was considerably higher in the negative controls than in the rats which had received a supplement of unsaturated acid.

(2) Whatever the diet, the proportion of the weights of saturated and unsaturated acids remained extraordinarily constant, the ratio being 38 : 62. Sinclair [1935] found that the proportion of saturated to unsaturated acids in the rat liver phospholipin fraction was 33·6 : 60·9.

(3) The iodine value of the unsaturated acids varied greatly, that of the acids from the negative controls being much lower than that from those rats which had received the doses of the esters of the unsaturated acids.

The rats which had been fed with the linolenic ester or with linseed oil contained the most highly unsaturated fats in their livers. This may possibly be explained by the fact that the dose of linoleate fed to Group II of the rats was known to be well below the optimum and that in the case of the rats dosed with the cod liver oil acid, the dose had ceased a fortnight before the rats were killed.

After the iodine value had been determined, each unsaturated fraction was brominated in ether solution at 0°; after standing overnight in the cold room, the solid ether-insoluble bromides were separated, washed well with ether, extracted with benzene, dried to constant weight and the bromine was estimated. The original weight of unsaturated acid present in the ether-insoluble bromides was then calculated. After the ether-soluble bromides had been weighed they were divided into a liquid fraction soluble in light petroleum and a solid residue small in amount and insoluble in light petroleum. The percentage of bromine was then determined in the petroleum-soluble fraction and the corresponding weight of unsaturated acid calculated. These results are set forth in Table II.

Table II

Supplement to fat-deficient diet	Wt. acids bromi-nated g.	Ether-insoluble bromides			Total wt. g.	Ether-soluble bromides						% of total acids (calculated from % Br in bromides)			
						(a) Petroleum-insoluble			(b) Petroleum-soluble liquids				Petroleum		
		Wt. g.	M.P. ° C.	% Br.		Wt. g.	M.P. ° C.	% Br	Wt. g.	% Br	Ether insol.	Insol.	Sol.	Total
Group I: None	0·2576	(a) Benzene-soluble 0·0264	203–4	61·8	0·41	0·04	190–5	—	0·368	39·76	3·8	5·9[1]	86·0	95·7
Group II: Methyl linoleate	0·2459	(b) Benzene-insoluble 0·0376	226–30[2]	64·3	0·43	0·07	200–5	—	0·363	44·78	5·3	10·9[1]	84·3	100·5
Group III: Methyl linolenate	0·1406	0·0831	Decomp.[2] above 260	69·0	0·26	0·11	145–8 with decomp.	—	0·149	45·94	17·6	28·7[2]	57·0	103·3
Group IV: Linseed oil	0·6506	0·2287	Decomp.[2] above 260	—	1·13	0·27	140–5 with decomp.	—	0·859	42·96	—	15·2[2]	57·0	—
Group V: Methyl ester of cod liver oil acid	0·2450	0·0908	Decomp.[2] above 260	70·76	0·41	0·14	—	62·3	0·263	39·1	16·1	21·6	67·0	104·7

[1] Calculated on the assumption that the bromides contain 61·8 % Br.
[2] Calculated on the assumption that the bromides contain 63·3 % Br.
[3] Traces of bromide of similar M.P. were found in the benzene extract.
Theory for decabromodocosanic acid: 70·8 % Br.
Theory for octabromodocosanic acid: 65·8 % Br.
Theory for octabromoarachidonic acid: 67·7 % Br.
Theory for hexabromoarachidonic acid: 61·06 % Br.
Theory for hexabromostearic acid: 63·31 % Br.

The nature of the unsaturated acids present in the liver

(1) *Group of negative controls.* The solid ether-insoluble bromide prepared from the negative controls was completely soluble in hot benzene and octa- or deca-bromides of acids containing 20 or 22 carbon atoms were therefore absent. The benzene-soluble bromide melting sharply at 203–4° does not seem to have been previously described. Linolenic hexabromide (M.P. 182–4°) was not present.

Analysis agreed with the formula $C_{20}H_{34}O_2Br_6$. (Found: Br, 61·80; C, 29·82; H, 3·97%. $C_{20}H_{34}O_2Br_6$ requires Br, 61·06; C, 30·54; H, 4·32%.) The bromide must, therefore, be derived from a C_{20} acid containing three ethylene linkages, possibly a dihydroarachidonic acid, but arachidonic acid itself is absent.

Sinclair [1935] separated the phospholipins from the total fat contained in the carcasses of rats fed on a fat-deficient diet and found that the ether-insoluble bromides contained 62·2% Br, a number appreciably lower than that required for linolenic hexabromide (63·32% Br); he suggested that unsaturated acids hitherto unidentified might be present in these controls. Since Sinclair's results were obtained on the phospholipin fraction, it is probable that the new hexabromide may also have been derived from phospholipin acid. The solubility of Sinclair's bromide in benzene was not definitely stated, nor did he not record its M.P., so that it is not possible to compare further these two substances. In our experiments the acid in the benzene-soluble bromide formed only 3·8% of the total acids. A further 5·9% of what appeared to be a less pure specimen of the same acid separated from the petroleum-insoluble fraction of the ether-soluble bromides. After recrystallization this melted at 190–195° but there was insufficient for analysis. In all, therefore, probably rather more than 9% of the total fatty acid consisted of the dihydroarachidonic acid. The ether-soluble fraction contained bromide corresponding with 86% of the total acids. The Br content was 39·76%, so that in addition to oleic dibromide (36·19% Br) some small proportion of liquid bromides of the more unsaturated acids must have been present.

There was no indication of the presence of either linoleic or linolenic acid.

(2) *Group fed with methyl linoleate.* Here the liver fatty acids yielded ether-insoluble bromides containing 64·3% Br corresponding to a yield of 5·3% higher unsaturated fatty acids. The bromide was insoluble in benzene; M.P. 226–30° with decomposition. From the ether-soluble bromide fraction a solid residue was obtained insoluble in light petroleum; when recrystallized, it melted at 200–205° and was probably therefore the dihydroarachidonic acid hexabromide isolated from the negative control; there was insufficient for analysis. The bromine percentage (64·3) in the benzene-insoluble bromide was less than that required by arachidonic octabromide (67·7). Since this fraction melted with decomposition at 226° it was possibly a mixture of arachidonic octabromide and dihydroarachidonic hexabromide, though if present the latter might have been expected to have been dissolved by the benzene. A somewhat similar fraction was obtained by Klenk & Schoenebeck [1932] in separating the acids of the phospholipins of ox liver. This melted with decomposition at 230–33° and by debromination and subsequent dehydrogenation was shown to consist of a mixture of C_{18} and C_{20} derivatives. It seems likely that a mixture of arachidonic acid and its dihydro derivative would behave similarly. Klenk and Schoenebeck identified considerable quantities of linoleic acid as the tetrabromide (M.P. 114–15°) but no evidence of this was found in the fat we examined. The separation of a bromide fraction insoluble in benzene and containing a higher % of Br than that in the C_{20} hexabromide indicate the presence of an acid containing four or more double bonds. As has already been pointed out, only minimal doses of linoleate had been fed to the rats in this group

and it is desirable that the fat should be investigated when adequate supplements of this acid were added to the fat-free diet.

The proportion of unsaturated acid derived from the ether-soluble bromide was 84·3 % of the total amount, in this respect closely resembling the state of affairs existing in the fat of the negative controls. The bromine content of the ether-soluble bromide was, however, higher (44·78 %), resembling that in the linolenic-fed rats.

(3) *Group of rats fed with methyl linolenate.* Here the amount of bromide insoluble in both ether and benzene corresponded to 17·6 % of the total acids. The bromide decomposed above 260° and contained 69·0 % Br; this would be in agreement with a mixture of arachidonic octabromide (67·7 % Br) and the deca-bromide of the C_{22} acid (70·8 % Br), or possibly a mixture of the octa- and deca-bromides of the C_{22} acid was present. The ether-soluble bromide fraction contained 45·94 % Br and corresponded to a mixture of oleic dibromide with liquid bromides of more unsaturated acids, the corresponding acids forming 57 % of the total. The petroleum-insoluble fraction of the ether-soluble bromides gave an indefinite M.P. at 145–8°; the bromide was not estimated but the corresponding acids probably formed more than 20 % of the whole amount present. Since linolenic hexabromide melts at 182–4°, the presence of an impure specimen of this is not excluded. Great caution has to be exercised in drawing deductions as to the nature of a bromide from its solubility when a large proportion of liquid bromides is also present in the solution.

(4) *Group fed with linseed oil.* Like that from the linolenic-fed rats, the benzene-insoluble bromide decomposed above 260° but unfortunately this fraction was accidentally lost before its weight and its Br content had been determined. The ether-soluble fraction contained 42·96 % Br and the corresponding acid formed 57 % of the total. There was also a fraction insoluble in light petroleum melting at 140–5°, the corresponding acid forming from 10 to 20 % of the whole. These results follow closely those obtained from the rats fed with linolenic acid.

(5) *Group fed with the methyl ester of the cod liver oil acid.* The benzene-insoluble bromide decomposed above 260°; analysis agreed with the formula for the bromide of docosapentaenoic acid $C_{22}H_{34}O_2Br_{10}$. (Found: C, 22·57; H, 3·01; Br, 70·8 %. Theory: C, 23·36; H, 2·72; Br, 70·8 %.) The free acid represented 16 % of the total fatty acids. The ether-soluble fraction also consisted of oleic dibromide with a small proportion of the bromides of the higher unsaturated acids, the latter forming 67 % of the total acids. The petroleum-insoluble fraction was a sticky solid containing 62·30 % Br; the debrominated acid therefore formed 21·6 % of the total.

DISCUSSION

The absence of any acid containing 20 or 22 carbon atoms and four or more ethylene linkages in the rats fed on the completely fat-free diet suggests that unless linoleic or linolenic acid is given, the rat-liver is unable to synthesize the C_{20} or C_{22} acid containing four or more double bonds, essential for the continued existence of the animal. The source of the dihydroarachidonic acid isolated from the livers of the negative controls is not certain. It has been established, largely owing to the work of Sinclair, that the replacement of these highly unsaturated acids takes place extremely slowly, and this is corroborated by the long time that is necessary to establish the symptoms of the fat deficiency disease in rats which had been placed on the fat-free diet immediately after thay had been weaned. The dihydroarachidonic acid was, therefore, probably derived from arachidonic

acid originally present in the rat, its synthesis from the oleic or saturated acids which the rat may still be able to form from carbohydrate is not excluded.

It was unfortunate that the only linoleate-fed rats available were those which had been kept for a very long time on the fat-free diet and then fed for 5 weeks with quite inadequate amounts of linoleate, so that they could not be expected to show typical results. Small as were the doses of linoleate, they resulted in the deposition in the liver of an acid containing more than three double bonds. From the bromine content of this bromide fraction, a mixture of arachidonic and dihydroarachidonic acids may have been present. Possibly because of the insufficiency of the doses of linoleate which had been given, the C_{20} hexabromide, which had been isolated from the negative controls, appeared to be present in the petroleum-insoluble fraction; presumably its bromide had been carried into solution by the large proportion of ether-soluble liquid bromides present.

There is a good deal of evidence in the literature that arachidonic acid is formed when oils containing linoleic acid are fed [Ellis & Isbell, 1926; Eckstein, 1929; Spadola & Ellis, 1936]. There is not, however, always a clear differentiation made between the octabromide of arachidonic acid and the decabromide of the C_{22} acid.

Again from the literature [Ellis & Isbell, 1926; Klenk, 1932; Snider & Bloor, 1933] convincing evidence is present showing that linolenic acid is not stored as such in the body. Our experiments seem to point to its conversion into the C_{22} acid with five double bonds. The function of the liver here is not to desaturate the 18-carbon chain but, given the suitable condition of desaturation, to build up a longer chain, additional double bonds being formed in the process. The classification of linoleic acid as a vitamin appears to us to be undesirable in view of the fact that linoleic and linolenic acids seem to be the necessary building stones for the synthesis of arachidonic and docosapentaenoic acids. The latter acid was also obtained from the livers of rats fed with the docosahexaenoic acid isolated from cod liver oil, so that in this the hydrogenation of one double bond must have occurred. This hexaenoic acid was shown [Hume et al. 1938] to produce no amelioration of skin symptoms; it is as yet unkown whether these are cured by arachidonic acid, since Turpeinen [1938], who carried out experiments on rats in which this acid was fed, gives only a general statement about the healing of skin symptoms.

It seems that whereas the essential linoleic or linolenic acids may have some direct effect on the skin, their main function is to supply material from which higher and more unsaturated acids may be synthesized. Sinclair [1935] has stressed the care with which these are guarded and the length of time that elapses before they are lost from the phospholipins which contain them, and has concluded that these are not, like other lipins, concerned in the transport of fat but play some special role in the animal metabolism. It is, however, of interest that in our negative controls where these acids were absent, the percentage of fatty acid in the livers was higher than in the livers where these more highly unsaturated acids were present. In the fat-deficiency disease there is an inability to store fat, but there is some evidence that fat may still be synthesized from carbohydrate; it seems possible that the highly unsaturated acids may play some part in the storage of fat, either by enabling the more saturated fats to be carried to the depots or by influencing its passage into the connective tissue cells in which it is stored. The study of the basal metabolism and respiratory quotients made by Wesson and Burr [Wesson, 1927; Wesson & Burr, 1931] lends some support to this view.

SUMMARY

The livers of rats fed on a fat-free diet were free from acids containing four or more double bonds: a hitherto unknown acid $C_{20}H_{34}O_2$ was isolated as its hexabromide, melting at 202–4°. Rats which had been kept for a long time on the fat-free diet and then fed with very small doses of methyl linoleate probably contained the same dihydroarachidonic acid as had been found in the negative controls. Arachidonic or some more highly unsaturated acid was also present and must, therefore, have been synthesized from the linoleic acid given.

Rats fed on a fat-free diet and then dosed with methyl linolenate (6 drops daily) synthesized an acid which, from the bromine content of its bromide, appears to have been a mixture of arachidonic and docosapentaenoic acids. The C_{20} trienoic acid was not detected. A fortnight after dosing with the C_{22} hexaenoic acid (prepared from cod liver oil) had ceased, the liver contained the C_{22} pentaenoic acid. Linoleic and linolenic acids appear to be the building stones essential for the production of more highly unsaturated acids which play some unknown part in enabling the animal to store fat in its depots and tissues.

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CCLXXXIII. THE ESTIMATION OF VITAMIN B$_1$ IN BLOOD

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MEIKLEJOHN [1937] has described a method, based upon the work of Schopfer [1935], of estimating vitamin B$_1$ in "small samples" (15 ml.) of blood. From his evidence he concludes "that the method...provides a quantitative estimate of the true vitamin B$_1$ content of the blood". Since a clinical test for vitamin B$_1$ in blood is badly needed, and since Meiklejohn's method is being used by several workers in this country and in others, it seemed to be important to examine the justification of his claim and the value of the test. The basis of the method lies in the ability of vitamin B$_1$ to promote the growth of a fungus, *Phycomyces blakesleeanus*.

There is already sufficient evidence contained in Meiklejohn's paper and in Schopfer's numerous publications to show that the former's claim needs investigation. Schopfer & Jung [1937, 2] have published tentative conclusions: "Le sang donne une réaction positive. En exprimant en aneurine la totalité de l'action auxogène observée, on arriverait à des teneurs variant entre 0·2 et 0·4γ par cm.^{3} de sérum." The animal used is not mentioned. In his other paper mentioning determinations on blood, Schopfer [1937] states: "Avec le sérum, nous avons trouvé une fois 39 mg. de récolte avec 1 ccm. (rat mâle) et une fois 69 mg. pour 1 ccm. (rat femelle); en comparant avec les données fournies par la vitamine pure, nous trouvons que 0·2γ de cette dernière livre une récolte de 65 mg." He then warns us of the danger of expressing all growth-promoting activity in terms of vitamin B$_1$, since "nous savons que d'autres facteurs agissent également". Although results on two rats hardly merit discussion, it is instructive to examine the only experimental results that he has published (presumably obtained with the male rat):

Rat serum vol. in ml.	Dry wt. of fungus mg.	Approx. value for apparent vitamin B$_1$ (γ, 100 ml.) calculated from Schopfer's figures
0·5	34	17
1·0	39	10
2·0	47	6·5
Rat red blood corpuscles "about 2 ml."	77	15

It will be noticed that 0·5 ml. and 1 ml. serum give about the same actual growth; 0·5 ml. is nearly three times as effective as 2 ml. if the results are expressed per unit volume. These meagre results indicate that the method cannot be used for accurate quantitative estimations. They also indicate that rat serum contains an inhibitory factor.

Further, the method is not specific for vitamin B$_1$. Schopfer had great difficulty in identifying the growth-promoting factor owing to its great stability to heat, resistance to alkali and solubility in chloroform. Because of these

properties he at times discarded vitamin B_1 as the growth factor and supported bios [Schopfer, 1932], vitamin D, flavin [Schopfer, 1934, 1] and "factor M" [Schopfer, 1934, 2]. The only substances now known to promote the growth of the fungus are vitamin B_1 and its breakdown products; the pyrimidine and thiazole fragments (and certain closely related compounds [Robbins & Kavanagh, 1938]) are active when they are supplied together [Schopfer & Jung, 1937, 1; Sinclair, 1937; Robbins & Kavanagh, 1937. It seems probable that "factor M" consists of these fragments; and since heating (e.g. in cooking) may destroy the vitamin, these substances may appear in blood and so invalidate the test [Schopfer & Müller, 1938].

Meiklejohn found that the blood of avitaminous pigeons had an adjuvant action on added vitamin. This fact strongly condemns the validity of the test, and it will be shown below that normal blood has a similar action.

Although these facts cast doubt upon Meiklejohn's claim, it seemed advisable to examine the test experimentally.

EXPERIMENTAL

Unless otherwise stated, the technique of these experiments has only differed from that described by Meiklejohn in a few particulars: 0·4 % asparagine[1] has been used unless otherwise stated; $MgSO_4$, $7H_2O$ in a concentration of 0·002 M was used instead of the anhydrous salt; the stock medium was brought to pH 6·5 with 10 N NaOH (the slight differences in pH produced by adding different amounts of blood did not affect the results), and was made up in stronger solution than used by Meiklejohn, so that only 4 ml. were added to each 50 ml. flask; 1 ml. spore suspension (containing about fifteen million spores) was used for inoculating. Since it has been found that the order in which the medium, blood, water and vitamin are added to the flask makes a difference to the results, unless otherwise stated the blood has been added to the medium and stood for at least half an hour; water (followed where necessary by vitamin) has then been added to the flasks and stood at $-2°$ for at least 12 hr. before sterilizing by steaming for 20 min. on 3 successive days. The cultures have been grown for 10 days in the dark in a room kept at a constant temperature of 18°. The stock culture of *Phycomyces blakesleeanus* (sex $-$) has been grown on Sabouraud's medium to which malt extract (B.P.) was added to make a concentration of 2 %. Most experiments have been set up at least in duplicate and results quoted are from typical experiments. Synthetic vitamin B_1 has been used throughout.

Composition of medium

In the method described by Meiklejohn, different concentrations of asparagine were used for the control flasks and for those containing blood. He found that in presence of 2 or 3 ml. blood, there might be some reduction of growth if the concentration of asparagine were increased above 0·2 %. This effect was said not to be observed with 1 ml. or less; one result with 1 ml. and none with less were quoted. The results with 2 and 3 ml. show an inhibition in the higher concentration of about 14 %. Most of Schopfer's experiments were done with 0·1 % asparagine, which is well below the optimum concentration. I have found that results with different concentrations of asparagine vary, but that 0·4 % with blood usually gives a greater growth than is given by 0·2 or 0·6 %. Even 0·4 % is suboptimal in the control flasks without blood (Table I).

Asparagine was the best source of nitrogen that Schopfer [1934, 3] tried, although glycine was almost as good. I have tested various compounds, using amounts which supplied the same quantity of nitrogen as is present in 0·4 % asparagine, and found that, as sources of nitrogen, guanidine sulphate, methyl

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[1] B.D.H. asparagine and dextrose (A.R.) were used; the two inorganic salts were supplied by Kahlbaum ("puriss.").

Table I. *Effect of concentration of asparagine on the growth of* Phycomyces

In this and all the other tables, unless otherwise stated, the figures
represent dry weight of fungus in mg.

Exp.		Concentration of asparagine (%)							
		0	0·1	0·2	0·3	0·4	0·6	0·8	1·0
1	0·1γ vit. B₁	0	—	30·8	—	41·0	38·4	40·2	—
	Ox blood 1 ml.	26·4	...	26·3	—-	27·9	23·5	25·3	—
	„ 2 ml.	51·6	—	52·4	—	52·6	48·4	45·3	—
2	Ox blood 1 ml.	27·6	—	35·2	—	35·4	40·1	40·0	—
	„ 2 ml.	—	—	61·4	—	65·3	69·2	79·3	—
3	0·1γ vit. B₁	0	- -	36·5	—	37·1	43·5	43·3	47·3
	0·5γ vit. B₁	0	—	73·8	—-	101·4	115·6	120·7	132·3
4	0·5γ vit. B₁	—	—-	—	—	101·0	113·8	—	—
	Ox blood 2 ml.	—	56·7	64·5	63·8	87·8	78·6	—	—
5	Ox blood 2 ml.	—	—	68·8	—	88·7	—	—	—
	+0·1γ vit. B₁	—	—	108·9	—	111·0	—	—	—
6	0·1γ vit. B₁	0	24·8	26·3	28·9	31·6	31·9	32·7	33·1
	0·5γ vit. B₁	0	38·1	60·0	66·8	93·7	100·9	101·2	98·4
	Ox blood 1 ml.	26·9	35·2	34·0	38·8	41·1	36·9	44·4	39·4
	+0·1γ vit. B₁	37·4	60·1	72·2	71·8	78·0	69·5	75·6	79·5
	+2·5γ vit. B₁	39·7	76·9	109·5	142·2	168·1	165·6	218·8	270·0
	Ox blood 3 ml.	43·9	66·4	86·3	92·4	100·2	93·4	97·5	97·2
	+0·1γ vit. B₁	52·6	81·4	104·2	110·7	126·9	115·8	124·0	128·2
	+2·5γ vit. B₁	59·5	88·5	135·6	169·7	195·5	219·3	233·5	309·3

guanidine HCl, dimethyl guanidine HCl, creatine and creatinine were useless;
ammonium carbonate, urea and glycocyamine were very poor; alanine and
arginine were poor; sodium aspartate and glycine were as good as asparagine,
and "glutamine" much more effective (the glutamine was added direct to the
medium, containing salts and glucose, before steaming; most of it will have been
destroyed by this process).

Table II. *Comparison of the effect of different sources of nitrogen
on the growth of* Phycomyces

			Source and % of nitrogen							
	Vit. B₁	Aspara-gine	Glycine			"Glutamine"				
Exp.	γ	0·4	0·2	0·5	0·75	0·2	0·4	0·6	0·8	1·0	1·2
1	0·1	46·0	46·0	50·7	53·8	43·8	53·2	56·2	—	—	—
	0·3	87·4	81·1	86·2	90·5	66·4	110·0	115·7	—	—	—
2	0·5	101·0	—	—	—	—	—	128·2	136·6	175·0	—
3	0·5	101·4	—	—	—	—	—	130·6	136·8	—	160·0

Glutamine was not quite as effective as asparagine when added to blood
(Table III).

Table III. *Comparison of the effect of different concentrations of asparagine
and "glutamine" in the presence of 2 ml. ox blood*

	Source and % of nitrogen				
	0·1	0·2	0·3	0·4	0·6
Asparagine	56·7	64·5	63·8	87·8	78·6
"Glutamine"	—	—	61·2	75·7	75·7

The most satisfactory source of nitrogen, either with or without blood, has been found to be hydrolysed casein.

100 g. caseinogen (Glaxo) were heated with 300 ml. conc. HCl and 300 ml. water under a reflux condenser for $3\frac{1}{4}$ hr. After bringing to pH 6·5 with NaOH the total volume was 950 ml.

Table IV. *Comparison of asparagine and hydrolysed casein as sources of nitrogen for* Phycomyces

Exp.		Aspara- gine 0·4%	Hydrolysed casein (%)							
			1	3	5	6	7	8	10	12
1	0·1γ vit. B_1	37·1	—	—	—	58·7	58·7	60·3	58·0	45·3
2	0·1γ vit. B_1	41·0	26·8	49·6	51·5	—	—	62·9	59·0	—
	1 ml. ox blood	27·9	—	53·1	55·8	—	54·1	66·3	63·3	—
	2 ml. „	52·6	—	86·3	105·0	—	102·6	110·8	82·1	—
	3 ml. „	70·0	—	81·7	86·7	—	88·0	84·3	81·8	—

Table IV and four other experiments have shown that with or without blood about 8% is the optimum concentration of hydrolysed casein. The growth obtained with this concentration and 0·1γ vitamin B_1 is about the same as is obtained with 0·4% asparagine and 0·2γ vitamin B_1. Without added vitamin, hydrolysed casein causes no growth of the fungus.

In Table IV the growth obtained with 3 ml. blood and hydrolysed casein is in general lower than that obtained with 2 ml. In Tables IV, V and VI a comparison is made of the growths obtained with and without blood or vitamin and the two different sources of nitrogen. From these and other similar experiments it is concluded that (i) in absence of blood, hydrolysed casein is a better source of nitrogen than asparagine; (ii) this result is also usually found in the presence of 1 ml. blood, although the estimated vitamin tends to be lower when hydrolysed casein is used; usually with 3 ml. blood, the estimated vitamin per unit volume of blood is less than with 1 ml. when hydrolysed casein is used, more than with 1 ml. when asparagine is used; (iii) when 0·1γ vitamin is added to blood, a greater growth than expected is usually produced in presence of asparagine, and a smaller growth than expected is produced in presence of hydrolysed casein; (iv) this adjuvant action of blood in presence of asparagine is very marked when excess vitamin is added; but with excess vitamin and hydrolysed casein, blood sometimes has an adjuvant action and sometimes an inhibitory effect. This inhibitory action is being further investigated. These experiments prove that part of the adjuvant action of blood in presence of asparagine is due to sources of nitrogen in the blood. In this connexion it is interesting to recall that Meiklejohn found that extracting the vitamin from blood by means of alcohol or removing protein by heating in acid solution resulted in a considerable loss of growth-promoting activity.

It must be stressed that Schopfer has made no attempt to choose, and has not claimed to have chosen, the best medium for this fungus. The one employed by him and by Meiklejohn is likely to be suboptimal since it is poor not only in sources of nitrogen but also in salts ($MgSO_4$ and KH_2PO_4 are the only salts added by Schopfer). The addition of small amounts of various salts to the medium increases the growth; Czapek-Dox salts produce a better growth, and addition of $CaCl_2$, $FeSO_4$, $CuSO_4$, $MnSO_4$ and pyrophosphate, all increase it to varying extents, as shown in Table VII. In all cases pure salts were used; they were brought to pH 6·5 and added before steaming.

Table V. *Comparison of the effects of asparagine, hydrolysed casein and blood as sources of nitrogen for Phycomyces*

Source of nitrogen: O = no added nitrogen; A = 0·4% asparagine; HC = 8% hydrolysed casein

Addition	Exp. 1 Human blood			Exp. 2 Ox blood			Exp. 3 Ox blood			Exp. 4 Human blood			Exp. 5 Human blood			Exp. 6 Ox blood	
	O	A	HC	O	A	HC	O	A	HC	O	A	HC	O	A	HC	A	HC
0·1γ vit. B$_1$	0	15·3	24·2	0	27·1	35·2	0	31·6	38·4	0	24·0	36·8	—	26·3	35·0	37·1	60·3
2·5γ vit. B$_1$	0	114·5	157·0	0	110·8	180·2	0	93·7	186·4	0	66·8	172·8	—	113·0	126·4	101·9	—
1 ml. blood	18·6	19·1	28·2	28·0	37·7	41·2	26·9	41·1	29·4	18·8	22·6	26·3	—	25·1	33·7	27·1	42·3
+0·1γ vit. B$_1$	32·0	44·6	43·0	36·1	66·1	66·5	37·4	78·0	49·4	—	63·1	—	—	48·0	—	—	—
+2·5γ vit. B$_1$	72·9	129·0	206·0	40·9	149·0	81·0	39·7	168·1	91·2	—	135·0	—	—	—	—	—	—
2 ml. blood				38·6	73·4	57·7				—	32·2	—	—	—	—	—	—
3 ml. blood	52·1	69·5	63·8	41·1	89·0	78·0	43·9	100·2	64·3	—	50·3	—	—	48·6	65·0	45·5	55·2
+0·1γ vit. B$_1$	63·5	90·8	81·8	—	—	—	52·6	126·9	77·6	—	—	—	—	63·0	—	—	—
+2·5γ vit. B$_1$	128·5	201·0	224·0	—	—	—	59·5	195·5	102·4	—	—	—	—	—	—	—	—

Table VI. *Comparison of the effects of asparagine and hydrolysed casein as sources of nitrogen for Phycomyces in presence of blood*

Vit. B$_1$ γ	Ox blood (ml.) 0	1	2	3	4	5	6	7
				Hydrolysed casein (8%)				
0	0	29·4	63·6	64·3	113·5	118·2	124·5	—
0·1	38·4	49·4	75·4	77·6	116·3	123·4	137·1	132·1
2·5	186·4	91·2	99·6	102·4	136·5	163·7	169·9	—
				Asparagine (0·4%)				
0	0	41·1	66·0	100·2	118·6	122·5	—	—
0·1	31·6	78·0	93·1	126·9	128·7	—	—	—
2·5	93·7	168·1	165·7	195·5	171·9	—	—	—

Table VII. *The effect of salts on the growth of* Phycomyces

Exp. 1	0·3 γ vitamin B_1	96·0
	0·3 γ vitamin B_1 + CaCl₂ (52 mg.)	131·5
	0·3 γ vitamin B_1 + sodium pyrophosphate (12 mg.)	116·2
Exp. 2	0·1 γ vitamin B_1	46·0
	0·1 γ vitamin B_1 + CaCl₂ (6 mg.)	53·4
	0·1 γ vitamin B_1 + CaCl₂ (50 mg.)	65·4
	0·1 γ vitamin B_1 + sodium pyrophosphate (1·7 mg.)	45·9
	0·1 γ vitamin B_1 + sodium pyrophosphate (8·0 mg.)	53·4
	0·3 γ vitamin B_1	87·4
	0·3 γ vitamin B_1 + CaCl₂ (6 mg.)	104·1
	0·3 γ vitamin B_1 + CaCl₂ (50 mg.)	126·2
Exp. 3	0·1 γ vitamin B_1	37·1
	0·1 γ vitamin B_1 + FeSO₄ (2 mg.)	41·1
	0·1 γ vitamin B_1 + CuSO₄ (2 mg.)	50·0
Exp. 4	0·1 γ vitamin B_1	39·5
	0·1 γ vitamin B_1 + MnSO₄ (1 mg.)	40·0
	0·4 γ vitamin B_1	83·0
	0·4 γ vitamin B_1 + MnSO₄ (1 mg.)	95·6

These salts were supplied in larger amounts than would be present in the blood added. But since the medium selected is not a good one for the growth of the fungus, it would be expected that there might be substances present in blood that would have an adjuvant action upon it. Experiments now to be described show that such is the case.

Other substances in blood affecting growth

Meiklejohn concluded that the presence of blood does not usually alter the activity of the vitamin. The three lines of evidence that he advances are not convincing.

(1) He states that adding known amounts of vitamin to blood that has been autoclaved at pH 9 gives the expected growth, and the four figures he quotes support this. Yet this treatment, as he points out, is so drastic that it destroys other substances; for instance the reaction shifts during the process to pH 5. It is shown below that the adjuvant factor is thermolabile.

(2) He states that "the addition of a small amount of vitamin B_1 usually increases the growth of mycelium to the same extent in the presence of added blood as in its absence". Results on three samples of 2 ml. blood with and without 0·1 γ vitamin are quoted in support of this. As already mentioned, he found that the blood from avitaminous pigeons had an adjuvant action on added vitamin and this I have confirmed. He stated that it was important to be aware of the possibility of blood containing an adjuvant factor, "and if necessary to control it" by including in the test a 2 ml. sample of blood to which 0·1 γ vitamin had been added. He says that this gives a growth "considerably less" than the maximum, but his own figures show that it may be equal to the growth obtained with excess vitamin B_1.

As this point is important, I have examined it in some detail, and find that blood always contains an adjuvant factor. In experiments with ox blood summarized in Table VIII, known amounts of vitamin B_1 were added to the flasks and the total vitamin content was estimated. In some cases it was possible to estimate the apparent vitamin B_1 in the blood by subtracting the amount of vitamin B_1 added from the estimated total vitamin content; in such cases the apparent vitamin B_1 content, expressed in γ/100 ml. blood, has been printed in

Table VII in ordinary type. In other cases the amount of growth was greater than that obtained even with excess of added vitamin in the absence of blood, so that it was not possible to translate the weights of growth into apparent amounts of vitamin B_1; when this was the case the actual weights of growth have been given and have been printed in black type, as have also the maximum growths obtained with excess of vitamin alone.

The three experiments illustrated in Table VIII and many others show that adding small amounts of vitamin to ox blood produces in about 75 % of cases a

Table VIII. *Effect of added vitamin B_1 on the estimated vitamin in blood*

(For explanation of figures, see text.)

Vit. B_1 γ	Ox blood (ml.)																
	0	0·5	1	2	3	4	5		0	1	2	3	4		0	1	2	3	4
0	0	11·0	8·0	9·0	10·5	132	139		0	10·0	11·0	110	132		0	7·5	8·0	13·5	11·5
0·025	—	—	—	—	—	—	—		—	11·0	13·0	114	131		—	—	—	—	—
0·05	—	9·0	10·0	10·5	9·5	137	166		—	11·5	19·0	117	138		—	9·0	11·0	—	—
0·1	—	6·0	8·5	9·0	9·5	140	—		—	16·0	108	134	139		—	11·5	16·0	—	—
0·15	—	8·5	13·0	8·5	—	—	—		—	31·0	—	—	—		—	—	—	—	—
0·2	—	15·0	18·0	7·0	129	—	—		—	109	129	135	—		—	—	—	—	—
Excess (2·5–5·0)	118	136	132	147	157	175	205		105	132	143	—	—		104	166	204	266	278
			Exp. 1							Exp. 2						Exp. 3			

greater growth than would be expected from the amount of vitamin added. The blood itself therefore usually has an adjuvant effect upon the growth of the fungus apart from the vitamin it contains.

Experiments with human blood have given similar results. In 150 1 ml. samples of blood from different cases the addition of $0·1\gamma$ vitamin B_1 has produced an increase over the expected of 50 % or more in 63 % of cases, an increase of less than 50 % or no difference in 30 %, and a diminution in 7 %. From this it appears that with small amounts of vitamin there is usually an adjuvant factor in blood, but occasionally an inhibitory factor.

One simple method of testing the validity of the method has not been used by Schopfer and has been disparaged by Meiklejohn. If there is no substance in blood other than vitamin B_1 which affects the growth of the fungus, then the growths obtained with and without blood when excess vitamin B_1 is added should be the same. They are not. In 50 experiments at least $2·5\gamma$ vitamin B_1 have been added to the flasks with and without the addition of different samples of human blood (1 ml.): this amount of vitamin is excessive because under the conditions of the experiments a maximum effect is obtained with about $0·5\gamma$. The flasks containing the blood have always produced a growth that is greater than the control; the increase varies from 14 to 160 %, with an average for the fifty of 78 %. Further figures will be found in Tables I, V, VI, VIII and XI. Meiklejohn stated that "when large amounts (e.g. $0·4\gamma$) of vitamin have been added [to blood] the effect has been less satisfactory. As noted previously, however, anomalous results are frequently obtained when the amount of vitamin is sufficient to produce nearly maximum growth." The size of the vessel and the accumulation of waste products of metabolism, amongst other factors, are suggested by him as probably explaining his inconsistent results. However, the results that I have quoted are consistent and significant and show that vitamin B_1 is not the only substance in blood that affects the growth of the fungus, since the addition of blood always produces a greater growth in presence of excess vitamin. An unusually large adjuvant action was shown by blood taken shortly after death

from a patient with aplastic anaemia; the following weights of fungus were produced:

	mg.
0·1 γ vitamin B$_1$	25·8
2·5 γ vitamin B$_1$	65·8
1 ml. blood	61·5
1 ml. blood + 0·1 γ vitamin B$_1$	90·7
1 ml. blood + 2·5 γ vitamin B$_1$	193·6

Meiklejohn's inconsistencies with nearly maximal growth are possibly partly due to insufficient drying of the mycelium before weighing; the mycelium takes a long time at 110° to reach a constant weight. Further, the adjuvant factor is thermolabile, and slight differences in the temperature at which the flasks are sterilized make considerable differences to the growth. With these precautions in mind, I have obtained consistent results.

Another fact that proves that blood has an adjuvant action is that large samples (more than 3 ml.) of blood (to which no vitamin has been added) usually give a growth that is greater than that obtained with excess vitamin B$_1$ (see Tables VI and VIII).

(3) The third line of evidence advanced by Meiklejohn to show that blood does not usually contain an adjuvant factor is that with separate samples of 1, 2 and 3 ml. blood the values obtained are in the ratio 1:2:3. He quoted six selected cases of human blood in support of this and states that he discarded the figures when this result was not obtained. I have found that there is usually an adjuvant factor present in blood so that the 3 ml. sample gives a value of more than three times the 1 ml. sample. Very rarely the blood has a depressant effect. Figures for ox blood have already been quoted. Some selected figures for human blood are as follows:

Table IX. *The effect of different volumes of blood upon the estimated vitamin in blood*

Apparent vitamin B$_1$ in blood (γ/100 ml.)

Ml. blood	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8
1	13	8	6	7·5	8	6	10	13
2	18	18	11	15	12	12	9	6·5
3	113	107	17	17	15	17	6	0
	(5 γ gives 106)	(2·5 γ gives 78)						

The figures in black type represent actual weights of fungus obtained (cf. Table VIII). Determinations upon samples of human blood from 235 different cases have given the following results: in 155 samples, the 3 ml. value (expressed in γ/100 ml. blood) has been 1 γ, or more, higher than the 1 ml. value, and in 38 samples 1 γ, or more, lower; in 213 samples the average 3 ml. value (expressed per unit volume) was 23 % higher than the average 1 ml. value, and in 22 samples the growth with 3 ml. blood added was greater than that given by excess vitamin. It must be admitted therefore that blood usually has an adjuvant action on the growth of the fungus; rarely there is an inhibitory action. This proves that Meiklejohn's method does not provide a quantitative estimate of the true vitamin B$_1$ content of blood.

The nature of this adjuvant factor is not fully known. It has already been shown that one cause of it is the additional sources of nitrogen, and possibly the salts, of blood. A further cause of it is to be found in the buffering power of blood. The fungus, in presence of excess vitamin, stops growing mainly because of the accumulation of products of metabolism. Although the medium is buffered

Table X. *The effect of calcium carbonate on the growth of* Phycomyces

A. Ox blood

		ml. blood			
Exp.		1	2	3	4
1	Blood alone	35·0	64·5	93·0	—
	+ CaCO$_3$	49·3	83·1	81·3	—
2	Blood alone	40·9	57·8	88·4	—
	+ CaCO$_3$	55·3	72·2	122·7	—
3	Blood alone	44·1	79·6	96·8	105·0
	+ CaCO$_3$	38·4	64·5	112·4	124·6
4	Blood alone	22·8	59·3	93·2	—
	+ CaCO$_3$	24·8	43·8	84·7	—
5	Blood alone	28·3	39·0	54·5	—
	+ CaCO$_3$	29·4	44·9	—	—
6	Blood alone	26·8	43·6	70·0	—
	+ CaCO$_3$	43·3	64·2	—	—
	+ CaCO$_3$ (added after steaming)	29·4	49·0	...	—
7	No vitamin B$_1$ {Blood alone	30·9	59·8	96·0	—
	+ CaCO$_3$	36·3	57·5	88·9	—
	0·1 γ vitamin B$_1$ {Blood alone	59·3	82·8	110·4	—
	+ CaCO$_3$	66·6	94·2	98·9	—
	2·5 γ vitamin B$_1$ {Blood alone	132·3	146·8	147·0	—
	+ CaCO$_3$	122·7	136·3	39·8	—

B. Human blood (1 ml.)

Exp.		
1	Blood alone	20·5
	+ CaCO$_3$	31·0
2	Blood alone	19·2
	+ CaCO$_3$	22·8
3	Blood alone	29·8
	+ CaCO$_3$	40·0
4	Blood alone	26·1
	+ CaCO$_3$	40·2
5	Blood alone	27·6
	+ CaCO$_3$	21·8

C. Without blood

	Vitamin B$_1$ (γ)					
	0·0125	0·05	0·1	0·5	2·5	4·0
Control	—	—	22·9	·	—	103·9
+ CaCO$_3$	—	—	43·9	—	--	164·2
Control	—	17·7	32·1	73·5	107·1	—
+ CaCO$_3$	—	22·4	38·9	80·8	120·8	—

	Vitamin B$_1$ (γ)				
	0·1	0·2	0·3	0·5	2·5
Control	45·1	70	87·5	113	—
+ CaCO$_3$	48·6	78	103·5	123·5	—
Control	33·7	61·5	84·5	118	140
+ CaCO$_3$	51·4	75	133	162	285
Control	34·2	—	—	—	115
+ CaCO$_3$	47·0	—	—	—	161
+ CaCO$_3$ (added after steaming)	42·5	—	—	—	135·5

with phosphate, the solution has a pH of about 2 at this time. When blood is present the final pH is between 4 and 5. Blood therefore buffers the medium and allows the fungus to continue to grow for longer than it would otherwise do. This is undoubtedly one factor that contributes to the adjuvant effect. It can be tested by adding excess $CaCO_3$ (about 200 mg.). This does not alter the original pH of the medium; free acids produced by the fungus are neutralized by the $CaCO_3$ and the pH of the medium when the fungus has ceased to grow is still 6·5. When this is done it is found that a greatly increased weight of mycelium is produced (Table X c). The technique can be applied to blood, although the original pH must be carefully controlled, particularly if excess oxalate was used as an anti-coagulant. As a result of a number of experiments it has been found that the addition of $CaCO_3$ usually, but not always, produces an increase (averaging 16 %) in the growth produced by blood; but it invariably produces an increase (36 % on the average) without blood, showing that the buffering of blood is responsible for part of the adjuvant action (see Table X). This is very important, because Meiklejohn believed that over the steep part of the growth/vitamin curve (i.e. with concentrations of vitamin below about 0·3γ/10 ml.) "the limit to growth is determined solely by the amount of vitamin present": the results summarized above show that his conclusion is not justified. It is interesting that pyruvic acid (isolated as the 2:4 dinitrophenylhydrazone) accumulates in large amounts in the flasks containing small amounts of vitamin (e.g. 0·1γ), but there is practically none in the flasks containing excess vitamin. This indicates that the vitamin may be concerned with the removal of pyruvate as much in the fungus as it is in the animal body.
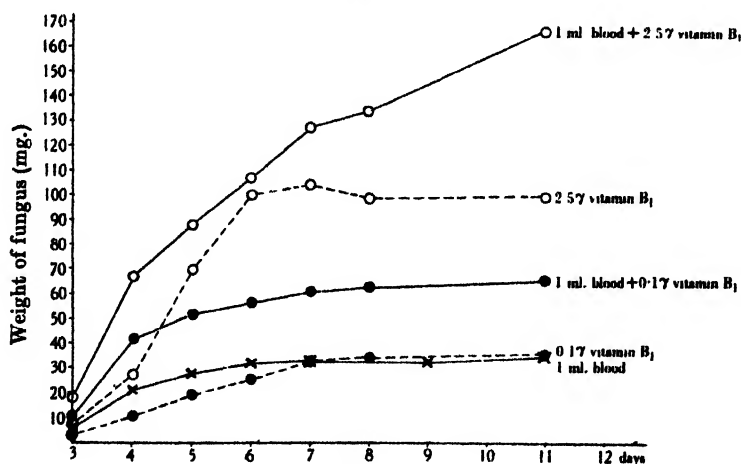
Fig. 1.

The growth produced in presence of blood differs in two further respects from the controls. First, the fungus in the flasks containing blood starts to grow more quickly than that in the control flasks containing approximately the same concentration of vitamin; the difference is particularly marked if vitamin be added to the blood (Fig. 1). Secondly, the ratio wt. of aerial hyphae/total wt. of fungus is much less in the flasks containing blood than in the control flasks containing approximately the same concentration of vitamin; and when excess vitamin is added to blood a very pale growth is produced which contrasts strongly

with the green hyphae in the control flasks with excess vitamin. These facts show that blood contains substances other than vitamin B$_1$ that affect the growth of the fungus.

There is one further piece of evidence that proves the existence of an adjuvant factor in blood. Meiklejohn stated that "some loss of growth-promoting activity has been found if the temperature has been raised above 110° or the time, at 107°, increased to more than 15 min." This presumably is meant to apply only in cases in which blood has been added, since the flasks without blood can stand autoclaving at higher temperatures for longer times without loss of activity. Vitamin B$_1$ is fairly stable at pH 6·5, and even if autoclaved for 10 min. at pH 8— which will undoubtedly cause some destruction—there is no loss in activity as Table III in Meiklejohn's paper shows. Schopfer & Jung [1937, 1] and Sinclair [1937] have indicated the reason for this, namely, that the vitamin is probably broken down to its constituent parts, and these can be utilized by the fungus. Schopfer & Müller [1938] have in consequence stated that it is necessary, when assaying vitamin B$_1$ with *Phycomyces*, first to adsorb the vitamin on fuller's earth and then to determine the vitamin in eluates made from this adsorbate. The loss of activity in the presence of blood which occurs on autoclaving at 107° (pH 6·5) for longer than 15 min. may be due to destruction of the vitamin but is more probably due to destruction of an adjuvant factor present in blood.

The results recorded in Table XI were obtained with human blood; the flasks were filled with medium and vitamin (where required), sterilized by steaming for 20 min. on three successive days and then filled under sterile conditions with the

Table XI. *The effect of heat upon the ability of blood to promote the growth of* Phycomyces

	Temperature (°)							
Addition	50	60	80	100	104·5	111·5	115·5	130·5
0·1 γ vit. B$_1$	—	—	—	15·3	15·0	15·5	15·2	12·3
2·5 γ vit. B$_1$	—	—	—	114·5	113·5	114·2	114·2	99·6
1 ml. blood	10·2	29·8	27·4	18·6	16·4	16·6	15·3	13·0
+0·1 γ vit. B$_1$	77·6	65·0	52·5	44·6	46·4	35·3	30·9	19·7
+2·5 γ vit. B$_1$	236·0	235·5	165·1	129·1	127·2	123·4	121·8	114·8
3 ml. blood	23·4	55·5	85·9	69·5	68·2	58·7	54·0	26·5
+0·1 γ vit. B$_1$	77·8	103·0	109·8	90·8	85·6	81·7	78·2	47·1
+2·5 γ vit. B$_1$	272·1	284·3	204·1	201·0	190·6	188·5	188·3	184·0

blood. The flasks were then heated at the required temperature for 10 min. I have already stated [Sinclair, 1938] that there is but little growth if the flasks containing blood are not heated because the vitamin in blood is bound; maximal growth is obtained after heating to about 60°; at higher temperatures there is a fall in growth due to destruction of adjuvant factor (shown by the fall in the flasks containing excess vitamin B$_1$). Since no change is produced by heating to 115° in the control flasks without blood, it is very important carefully to control the exact temperature in estimations; sterilizing at 110° for 10 min. will give a value about 15 % lower than that obtained by sterilizing by steaming and 5 % lower than after heating to 105°. This effect is also illustrated by Table XII.

Effect of storing blood

In any elaborate quantitative test, such as this, which is to be used for clinical purposes, it is often necessary to send samples of blood by post, or to keep samples until an experiment can be set up. (It may be mentioned that the

time consumed in the estimation on each sample of blood is about 2 hr. The test takes about a fortnight to complete.) Meiklejohn stated that oxalated blood will keep for "some hours" at room temperature without loss of vitamin; in presence of medium at $-2°$ it would keep "for days".

A few experiments have been done to test the effect of keeping the blood. The vitamin content was found to remain the same for about 6 days at $-2°$. If blood is kept for more than one week at this temperature the growth-promoting power usually increases owing to increase in adjuvant factor (see Table XII); this is probably due to autolysis producing compounds with adjuvant action. In seven experiments in which the blood was kept for between 8 and 24 days at $-2°$, the average increase in growth compared with the same sample tested at once was 32 % (16–50 %). In the experiment shown in Table XII human blood was drawn under sterile conditions. Some was added at once to the flasks containing medium etc. and sterilized next day (column I). Some was kept for 12 days at $-2°$ and then treated like the first sample (column II); it was also tested for sterility.

Table XII. *The effect of keeping blood upon its ability to promote the growth of* Phycomyces

		I	II
1 ml. blood	Unheated	—	8·5
„	Steamed	31·0	40·6
„	Autoclaved (110' for 10 min.)	27·4	36·5
1 ml. blood + 0·1 γ vitamin B_1	Steamed	60·0	58·7
1 ml. blood + 2·5 γ vitamin B_1	„	106·0	160·0
2 ml. blood	„	53·4	62·4
3 ml. blood	„	63·4	78·3

Specificity of the test

It has already been stated that the method is now known not to be specific for vitamin B_1. In his paper Meiklejohn reported tests for four substances possessing growth-promoting activity for other organisms with negative results. Two of his observations I have been unable to confirm.

(1) Meiklejohn stated that a specimen of bios supplied by Mr J. R. O'Brien was without effect. I obtained specimens of bios from the same source and found that they had a remarkable effect (see Table XIII).

Table XIII

	mg.
Bios (0·1 ml.)	170
Bios (0·1 ml.) + 0·1 γ vitamin B_1	164
4·0 γ vitamin B_1	104

It will be seen that bios (1 ml. = 21 mg. solids = 500 "doses") had a very much greater effect than excess vitamin B_1.

(2) Mannitol was stated by Meiklejohn to have no effect on the growth of the fungus. This confirmed the previous report by Schopfer [1934, 3].

(3) Knight's staphylococcal factor was stated to have only negligible activity in amounts of 100γ. Since Knight [1937] has shown that his factor consists partly of the breakdown products of vitamin B_1, and since three groups of workers [Schopfer & Jung, 1937, 1; Sinclair, 1937; Robbins & Kavanagh, 1937] have all found that these products are active in very small amounts, Meiklejohn's results are difficult to interpret. The remainder of Knight's factor is nicotinic acid or its amide; neither of these had any action on the growth of the

fungus. Coenzymes I and II, pure riboflavin and β-alanine were also inactive. A highly purified preparation of vitamin B_6 had no activity as a growth factor in the absence of vitamin B_1, but when added with the latter it doubled the growth.

In addition to bios, autoclaved "marmite", autolysed yeast and "Peters's eluate" all produced growths much greater than those obtained with excess vitamin B_1, even after they had been autoclaved at pH 9 to destroy this vitamin. I have already stated [Sinclair, 1937] that cocarboxylase is about as active as vitamin B_1, and under the conditions of the test the same is true of vitamin B_1 monophosphate. These results will be reported in detail later. It may be mentioned that several substances have been found that inhibit the growth of the fungus in small concentrations. For instance, 1 mg. indole in 10 ml. produces complete inhibition of growth.

Availability of vitamin in blood

In Meiklejohn's method, the corpuscles are allowed to settle into a thick mass at the bottom of the flask. Since he states that over 80 % of the vitamin in blood is associated with the corpuscles it is important to ensure that it all becomes available for the fungus. Meiklejohn advances three lines of evidence in support of this. The first is not satisfactory because, as shown above, the value obtained with 3 ml. blood is usually more than three times that obtained with 1 ml. and therefore the smaller relative growth which would be expected in the larger sample if some of the vitamin were not available would be masked by the adjuvant action. The second is not fully convincing: in one experiment only, the corpuscular layer was apparently autoclaved twice, and this, Meiklejohn states, caused some loss of activity; in any case, the difference in the growths given by the surface layer (27·5 mg.) and whole blood (33·0 mg.) was not large. The third statement—that the corpuscular layer at the end of an experiment possesses no growth-promoting activity—is not surprising, since the fungus is known to produce an inhibitory substance during growth which would presumably be present in the corpuscular layer.

The availability of the vitamin in the corpuscular layer can be simply tested by comparing laked blood with blood in which the corpuscles have been allowed to settle. The results of 24 experiments show that there is no significant difference between the two methods and therefore Meiklejohn's method of allowing the corpuscles to settle out is fully justified.

Since in the control flasks the medium is liquid and in the flasks to which the larger samples of blood have been added it is partly solid, the effect of the fluidity

Table XIV. *The effect of the fluidity of the medium on the growth of* Phycomyces

A. Ox blood

	Vitamin B₁ (γ)		
	0·1	2·5	1 ml. blood
Control	30·7	117·5	20·7
With agar	43·2	142·5	31·3

B. Human blood

	1	2	3	4 ml. blood
Control	27·4	63·7	96·7	110·0
With agar	39·4	67·0	87·5	114·1

of the medium was tested by adding agar (50 mg.) to some flasks. The addition caused a slight increase in growth (Table XIV) although agar without added vitamin or blood produced no growth. It appears therefore that the fungus grows rather better on a solid medium, and part of the adjuvant action of the blood may be due to this fact.

DISCUSSION

Williams, who originally [1919] suggested the use of yeast for the assay of vitamin B, has recently [1937] stated that "the use of fungi in quantitative testing for vitamin B_1 in extracts appears hazardous in the extreme". Orr-Ewing & Reader [1928] elaborated a quantitative test for vitamin B_1, using its growth-promoting activity with *Streptothrix corallinus*; the test was useful with fairly pure solutions of the vitamin, but interfering substances were present in cruder preparations. Heating in alkaline solution destroyed the vitamin, but some growth-promoting activity with the Streptothrix still remained [Peters *et al.* 1928]. Peters [1930] made the excellent suggestion that the micro-organism required the vitamin and could reactivate "alkalized" torulin. In these respects this micro-organism behaves like *Phycomyces*. In his earlier papers Schopfer realized that the fungus could only be used for the assay of fairly pure solutions of the vitamin. In his recent work he has become bolder and used the method with plant extracts, animal tissues, milk and blood; it has already been shown that his results on blood are not satisfactory. The fungus gives an appreciable growth with only 10^{-7}% vitamin B_1, and there is no doubt that Schopfer's method is the most sensitive one for assaying pure solutions of the vitamin. For this purpose it has proved very successful [Faguet, 1937; Villela, 1938].

But blood contains other substances that influence the growth of the fungus; this is not surprising when it is remembered that it contains "phytotoxins" [Macht, 1936]. The method is not specific; according to Schopfer & Jung [1937, 2], *Phycomyces* "nécessité pour sa culture en milieu synthétique, la présence d'une constellation de facteurs de croissance de nature vitaminique parmi lesquels se trouve la vitamine B_1". Therefore Schopfer & Müller [1938] state that the vitamin must be adsorbed on fuller's earth before it can be assayed by this method. Moreover, it has been shown above that blood has an adjuvant action upon the growth of the fungus under the experimental conditions of the test. It was shown that addition of small amounts (about 0.1γ) of vitamin to blood usually produced a growth greater than would be expected from the amount of vitamin added, that adding excess vitamin to blood invariably produced a growth greater than the control without blood, that large samples of blood (greater than 3 ml.) usually gave a growth greater than that obtained with excess vitamin and that the adjuvant action was partly destroyed by heat and usually increased when sterile blood was kept at $-2°$ for more than a week. The adjuvant action is probably due to more than one factor; sources of nitrogen and salts in the blood, the buffering power of the blood and the more solid medium produced when blood is added, particularly in large amounts, probably all contribute to it. Under certain conditions, blood may be shown to have an inhibitory action on the growth of the fungus. Probably the inhibitory factor is normally masked by the adjuvant factor and is only apparent when the adjuvant action of blood is diminished by such devices as improving the sources of nitrogen in the medium.

These facts prove that blood contains substances other than vitamin B_1 that influence the growth of *Phycomyces* under the conditions of the test. Meiklejohn's conclusion cannot therefore be substantiated. I agree with Van Veen [1937] that in testing impure extracts (including blood and urine) by this method "the

results were far from reliable; in fact, that they were at times entirely unserviceable". Yet this is the only method available at the present time for attempting to assay vitamin B_1 in blood. If the sources of error mentioned in this paper are borne in mind and are controlled as far as possible, then the method is valuable for comparing the apparent vitamin B_1 in different samples of blood.

SUMMARY

1. Following the work of Schopfer with the fungus *Phycomyces*, Meiklejohn described a method of estimating vitamin B_1 in blood. His conclusion that it provides a quantitative estimate of the true vitamin B_1 content of the blood has not been confirmed.

2. It is shown that under given conditions blood always contains substances that affect the growth of the fungus.

3. Hydrolysed casein is the most satisfactory source of nitrogen that has been found. By means of this it can be shown that sources of nitrogen in the blood affect the growth of the fungus even in presence of optimal concentrations of asparagine.

4. Under the conditions of the test the addition to blood of small amounts of vitamin B_1 usually produces a greater growth than expected, and addition of excess vitamin invariably does so.

5. The method is known not to be specific for vitamin B_1.

6. The importance is emphasized of carefully controlling factors such as the temperature of autoclaving and the length of time the blood is stored.

7. If the possible sources of error are borne in mind and controlled as far as possible, the method is valuable for comparing the apparent vitamin B_1 in different samples of blood.

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CCLXXXIV. WATER-SOLUBLE B VITAMINS

XI. THE ESTIMATION OF YEAST ELUATE FACTOR AND YEAST FILTRATE FACTOR BY RAT GROWTH METHODS

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IN this laboratory it has been shown that, in addition to riboflavin, at least two other components of the vitamin B_2 complex are required for the nutrition of the rat [Edgar & Macrae, 1937].

One of these components we have named yeast eluate factor, since it was adsorbed from autoclaved yeast extracts by fuller's earth and was found in barium hydroxide eluates prepared from the fuller's earth adsorbates. This factor we recognized primarily as a growth factor for rats although we also found it curative of rat dermatitis. Dr Lepkovsky kindly put at our disposal a sample of his pure factor 1 [Lepkovsky, 1938], which is required by rats for growth and prevention of dermatitis [Lepkovsky *et al.* 1936] and is identical with crystalline vitamin B_6 [György, 1938; Keresztesy & Stevens, 1938; Kuhn & Wendt, 1938].

We have found that crystalline factor 1 completely replaces our yeast eluate fraction in the diet of the rat. We have also isolated from yeast a substance identical with crystalline factor 1 [Edgar, El Sadr & Macrae, unpublished] and thus the identity of our yeast eluate factor with factor 1 and vitamin B_6 is proved. The name adermin has been suggested [Kuhn & Wendt, 1938] for the crystalline material.

The other component we have named yeast filtrate factor since it was present in the filtrate obtained by fuller's earth treatment of autoclaved yeast extracts. This factor is required for the growth of rats and, as far as that property is concerned, corresponds with the factor 2 of Lepkovsky *et al.* [1936]. It is uncertain if our factor is identical with that required for growth and prevention of dermatitis in chicks fed on a diet of heated grains [Elvehjem & Koehn, 1935; Lepkovsky & Jukes, 1935; 1936]. Neither nicotinic acid nor nicotinamide could replace the yeast filtrate fraction in the diet of the rat [Macrae & Edgar, 1937].

The work carried out in this laboratory during the past 2 years, which has included the chemical investigation of these fractions and the preparation of large amounts for other biological investigations, has required that we should have reliable methods for the detection and estimation of these factors.

Rat growth tests have been successfully used in this laboratory for the determination of aneurin (vitamin B_1) and riboflavin (lactoflavin). As well as these members of the vitamin B complex, yeast eluate factor and yeast filtrate factor profoundly affect the growth of young rats [Edgar & Macrae, 1937], and we have found it comparatively simple to devise rat growth tests, similar to those used to determine aneurin and riboflavin, which have proved satisfactory for the determination of yeast eluate factor and yeast filtrate factor. These experiments are described in the present paper.

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The general methods used in the tests for the B-vitamins have been described in previous papers from this laboratory [Chick et al. 1935; Edgar et al. 1937]. In the preparation of the litters of rats, the mothers received the full stock breeding diet except during the last week of lactation, when yeast was withheld. The number of rats in the litters was restricted to 8. The young rats, weaned at 21 days and weighing 40–50 g., received the basal diet consisting of casein 100, rice starch 300, cotton-seed oil 60, lard 15, salt mixture (McCollum's No. 185) 25 and water 500; the diet was cooked by steaming for 3 hr. Each rat also received from weaning daily supplements of 0·08 ml. cod liver oil and $10 \mu g$. aneurin; when the body weight of the animals reached 100 g. the daily supplements of cod liver oil and aneurin were increased to 0·1 ml. and $15 \mu g$. respectively.

In the preparation of vitamin B-free diets, the principal difficulty is to obtain vitamin-free protein of high nutritive value. We have found "Glaxo ashless extracted" casein to be satisfactory for certain investigations, although we have recognized that it is probably not free from B-vitamins. This casein was used in the earlier experiments reported in this paper, but later it was found advantageous to use a more highly purified casein.

Supplee et al. [1936] demonstrated that the washing of casein with a solution of NaCl removed riboflavin, and it is our experience that this process also removes other factors of the vitamin B complex from the casein. In the later experiments reported in this paper the casein used was purified by the following method.

"Glaxo ashless extracted" casein (2·5 kg.) was stirred for 30 min. with a solution containing 600 g. NaCl and 30 ml. glacial acetic acid in 30 l. tap water. The casein was allowed to settle for 1 hr. and the supernatant liquor was poured off. The washing with the salt solution was repeated 6 times. The casein was then pressed dry on Büchner funnels and stirred into 96 % alcohol (5 l.). The alcohol was removed by filtration and the casein spread in thin layers on the open bench and dried in a current of air.

The rats receiving the basal diet with supplements of cod liver oil and aneurin usually increased slightly in body weight during the first week after weaning, the average increase being about 4 g. for the period. The animals were then treated differently according to whether they were to be used in tests for eluate factor or filtrate factor. The animals were weighed 3 times weekly.

Preparation of supplementary materials

Yeast eluate fraction. The barium hydroxide eluate prepared from the fuller's earth adsorbate from autoclaved yeast extracts, purified by treatment with basic lead acetate, has proved thoroughly satisfactory. The method of preparation was described in a previous paper [Edgar & Macrae, 1937].

Yeast filtrate fraction. In the earlier experiments the filtrate fraction was obtained by simple treatment of an autoclaved yeast extract with fuller's earth by the method previously described [Edgar et al. 1937]. More recently we have purified this material by extraction with amyl alcohol, a procedure which has also proved effective in the purification of the factor required for the growth and prevention of dermatitis in chicks receiving a heated grain diet [Elvehjem & Koehn, 1935; Lepkovsky & Jukes, 1936]. The following method has been employed by us.

The fuller's earth filtrate (4 l., of concentration 1 ml. equivalent to 0·5 g. dry yeast) was concentrated in open trays at 37° to one-fourth of its original volume. H_2SO_4 was added to pH 1 and the extract was shaken with 2 l. amyl alcohol.

After separation, the amyl alcohol extract was shaken with 500 ml. water to which was added just enough NaOH to make the aqueous layer alkaline to thymol blue. The aqueous extract was separated, neutralized with HCl and the amyl alcohol again extracted with a second portion of alkali. The amyl alcohol was now transferred back to the yeast extract and the extraction repeated until the yeast filtrate had been extracted 6 times, the amyl alcohol being extracted with the alkali after each treatment of the yeast extract. The combined aqueous extracts of the amyl alcohol, containing the filtrate factor, were evaporated to small volume and treated with 4 volumes 96% alcohol. The precipitated salts were removed by filtration and the alcohol was distilled off *in vacuo*. A small amount of a gummy material which separated was discarded; in the final product 1 ml. was equivalent to 2 g. dry yeast.

Estimation of yeast eluate factor

Male rats were found more satisfactory for this test. They were prepared as described above and had received for 1 week from weaning the basal diet supplemented by cod liver oil and aneurin. For a period of 2 weeks the animals now received daily additional supplements of $50\,\mu g$. riboflavin and an amount of the yeast filtrate fraction equivalent to 2 g. dry yeast; these amounts were sufficient to supply the animals' requirements. An immediate growth response resulted and during the first week the animals usually increased in body weight by about 20 g. A slight fall in the rate of growth occurred in the second week during which the weight increase was usually about 15 g. Certain animals then received additional supplements of the materials to be tested for eluate factor activity, others (negative controls) were given no added supplement, while yet others (positive controls) were given an amount of a tested yeast eluate fraction equivalent to 2 g. dry yeast, this amount being known to supply the animals' requirements of eluate factor. The test was continued for a further 2-week period. Even after 2 days a sharp increase in the growth rate of the animals now receiving eluate fraction was noted, and the increased growth rate was maintained during the 2-week period, the average weekly gain usually being 25–30 g. (Fig. 1, Table I). The negative control animals generally continued to increase in weight at the rate observed towards the end of the preliminary period of 2 weeks, this rate being about half that of the positive control animals. Comparison of the growth rates of the animals receiving the test material with those of the negative and positive control animals indicated the vitamin potency of the tested material. As far as was practicable, animals used in a single test were taken from the same litter, although usually no great difference was observed from litter to litter. Reliable results were obtained when 3 animals, together with one positive and one negative control animal, were used for each test.

In the earlier experiment in which the basal diet contained unwashed "Glaxo ashless extracted" casein as source of protein and the unpurified yeast fuller's earth filtrate as source of filtrate factor, the slackening in the growth rate during the second week of the preliminary period was less marked than that observed when the more highly purified casein and purer filtrate factor preparations were introduced into the basal diet. The body-weight increase observed during the 2-week period following administration of eluate factor was also slightly less in the case of the animals receiving the purer diet; however, the growth response obtained when eluate factor was added to the diet was more striking and tests using the purified diet were generally more satisfactory.

It was observed that the increased growth rate resulting from the addition of eluate fraction varied with the dose given. In one experiment the average

total weight increase of 4 rats receiving a suboptimal dosage of eluate fraction was 40 g. for the 2-week test period, that of 5 rats receiving twice the above dosage was 49 g., while 4 rats given 4 times the dosage increased in body weight by 55 g. during the 2-week period. From this and other experiments not recorded it appeared that the growth response curve was the usual logarithmic one.
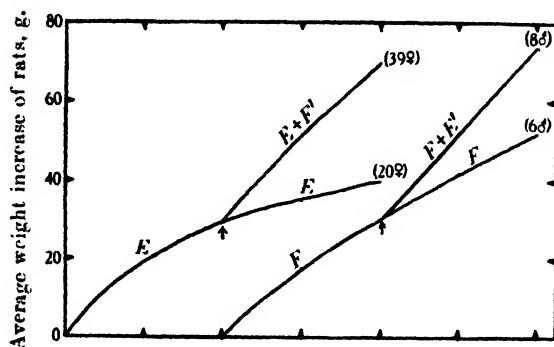
Fig. 1. Growth of young rats receiving a basal diet free from the B-vitamins, supplemented daily by 10–15 μg. aneurin, 50 μg. riboflavin and one or more of the following fractions: E = yeast eluate fraction (= 2 g. dry yeast), F = yeast filtrate fraction purified by amyl alcohol extraction (= 2 g. dry yeast), E' = eluate fraction from yeast (= 2 g. dry yeast), or liver (= 12 g. fresh liver), F' = filtrate fraction from yeast (= 2 g. dry yeast) or liver (= 6 g. fresh liver). The arrows indicate the points at which the doses were changed, and the figures and symbols in brackets the number and sex of the rats from which the growth curves were derived. Divisions on the abscissa indicate periods of 1 week.

Table 1. *Tests for eluate factor*

Each rat (male) received daily 10–15 μg. aneurin and 50 μg. riboflavin

No. of rats	Filtrate fraction, preparation and amount given daily	Av. weekly wt. increase of group for preliminary period of 2 weeks g.	Additional supplement given during test period	Av. weekly wt. increase of group during test period of 2 weeks g.
Unwashed casein diet:				
52	Yeast filtrate fraction (= 1 g. dry yeast)	21, 19	Eluate fractions from yeast or liver	29, 25*
36	„ „	21, 18	None	16, 16†
4	Yeast filtrate fraction purified by extraction with amyl alcohol (= 2 g. dry yeast)	21, 14	Eluate fractions from yeast or liver	27, 27
Washed casein diet:				
9	Yeast filtrate fraction (= 1 g. dry yeast)	19, 16	„	27, 24
6	„ „	20, 19	None	15, 15
8	Yeast filtrate fraction purified by extraction with amyl alcohol (= 2 g. dry yeast)	16, 12	Eluate fractions from yeast or liver	22, 22
6	„ „	19, 13	None	12, 10

* The standard error of the average total weight increase for the 2-week test period $(\sigma/\sqrt{n}) = 1\cdot08$.
† The standard error of the average total weight increase for the 2-week test period $(\sigma/\sqrt{n}) = 1\cdot29$.

The unit of eluate activity we have adopted is based on the potency of an amount of our standard eluate fraction equivalent to 2 g. dry yeast. This amount, when given daily to a rat prepared as described above, produces a growth response of approximately 90 % of the maximum obtainable.

Estimation of yeast filtrate factor

A method very similar to the above has been used for the determination of yeast filtrate factor. Rats of both sexes, prepared as described above, having received the basal diet supplemented only by aneurin and cod liver oil for one week from weaning, received daily for 2 weeks 50 μg. riboflavin and a dose of yeast eluate fraction equivalent to 2 g. dry yeast. By the end of the first week the animals had generally increased in weight by approximately 20 g.; during the second week of this period, however, a very marked slackening in the growth rate occurred, and by the end of the week the growth rate was usually about 7 g. weekly, although over the whole of the second week weight increases of about 10 g. were generally obtained. Certain animals then received the added supplement of the material to be tested for filtrate factor activity. Negative control animals were given no added supplement, while positive control animals received either the yeast fuller's earth filtrate fraction from 1 g. dry yeast or the preparation of filtrate fraction purified by extraction with amyl alcohol, equivalent to 2 g. dry yeast. The test proper lasted for 2 weeks, and the presence of filtrate factor in the test material was indicated by an immediate increase in the growth rate, unmistakable even within 2–3 days. The growth rate increased to about 22 g. weekly, and this was maintained for the 2-week period (Fig. 1, Table II). The negative control animals usually continued to increase in body weight at the rate of about 7 g. weekly. Reliable results were obtained when

Table II. *Tests for filtrate factor*

Each rat received daily 10–15 μg. aneurin, 50 μg. riboflavin and yeast eluate fraction (=2 g. dry yeast)

No. of rats	Sex	Av. weekly wt. increase of group for preliminary period of 2 weeks g.	Additional supplement given during test period	Av. weekly wt. increase of group during test period of 2 weeks g.
Unwashed casein diet:				
6	♂	22, 12	Filtrate fractions from yeast or liver	25, 26
3	♂	19, 10	None	7, 5
39	♀	20, 10	Filtrate fractions from yeast or liver	22, 19*
20	♀	18, 10	None	6, 5†
Washed casein diet:				
10	♂	18, 11	Filtrate fractions from yeast or liver	21, 19
14	♂	19, 9	None	9, 6
7	♀	19, 8	Filtrate fractions from yeast or liver	19, 16
20	♀	18, 8	None	5, 4

* The standard error of the average total weight increase for the 2-week test period $(\sigma/\sqrt{n}) = 0.88$.
† The standard error of the average total weight increase for the 2-week test period $(\sigma/\sqrt{n}) = 1.11$.

3 animals, exclusive of controls, were used for each test. As far as possible animals were taken from the same litter but we found little variation between the litters. A certain difference existed between the males and females and this had to be considered in planning the tests. The growth response varied with the

amount of filtrate fraction given (see Table III). The tests in which the purer casein was used were generally more satisfactory, as the growth rate diminished more regularly during the second week of preparation when the animals were receiving the riboflavin and eluate factor supplements.

Table III. *Growth response of rats to graded doses of filtrate factor as contained in amyl alcohol extracts from* (a) *yeast filtrate fractions, and* (b) *liver filtrate fractions*

Each rat (female) received daily 10–15 μg. aneurin, 50 μg. riboflavin and yeast eluate fraction (= 2 g. dry yeast)

	No. of rats	Av. weekly wt. increase of group for preliminary period of 2 weeks g.	Daily filtrate supplement given during test period	Av. weekly wt. increase of group during test period of 2 weeks g.
(a)	4	23, 12	Equiv. of 1 g. dry yeast	14, 20
	3	22, 11	Equiv. of 2 g. dry yeast	19, 22
	2	23, 13	Equiv. of 6 g. dry yeast	22, 18
(b)	2	17, 5	Equiv. of 3 g. fresh liver	11, 13
	2	22, 9	Equiv. of 6 g. fresh liver	23, 18

The unit of filtrate factor activity we have adopted is based on the potency of an amount of our standard filtrate fraction, purified by amyl alcohol extraction, equivalent to 2 g. dry yeast. This amount, when given daily to a rat prepared as above, produces a growth response of approximately 90 % of the maximum.

DISCUSSION

The rat growth method for the determination of factors of the vitamin B complex has been so extensively used in the past that the use of this criterion for the study of new factors of the vitamin B complex requires no further justification. In the case of aneurin, the rat growth method, although perhaps more tedious than some others, is certainly one of the more reliable biological methods for the determination of that vitamin. The biological determination of riboflavin has been almost exclusively carried out by rat growth methods; a paper published from this laboratory [Edgar et al. 1937] showed the regular growth response of young rats deprived of riboflavin to administered doses of that substance.

Although curative methods for vitamin determination have the advantage of specificity in cases where a specific group of symptoms is involved, growth methods which employ uniform healthy young animals appear to us to be the more reliable. Curative methods necessitate the use of sick animals and the response effected by administration of the missing vitamin is largely dependent on the degree to which the pre-existing deficiency had affected the general health of the animal.

For the estimation of vitamin B_6, which we now realize is identical with our yeast eluate factor and factor 1 [Lepkovsky et al. 1936], the cure of the dermatitis developed by rats deprived of the vitamin has been most extensively used [György, 1935]. In our laboratory, when using this method, we have encountered the disadvantages of curative methods referred to above. Further, we have also noted occasional spontaneous cures in our animals and have also found that the specificity of yeast eluate factor for the cure of this dermatitis is not complete [Chick & Macrae, unpublished]. Cures of dermatitis developed on diets deficient in yeast eluate factor and riboflavin, or deficient in yeast eluate factor and yeast

filtrate factor have frequently been obtained by the addition of riboflavin or of yeast filtrate factor, respectively. In carrying out the rat curative test, therefore, the supply to the animal of all the other vitamin B factors must be adequate. Since the animals used in curative tests are usually on experiment for many weeks, during which they must be carefully observed, the time required for these tests is much greater than the simple routine rat growth test described.

The only biological property of our yeast filtrate factor at present recognized is its effect on the growth rate of young rats. It is possible that the factor required by chicks for growth and prevention of dermatitis may be identical with this rat factor and, if this is found to be the case, the determination by the chick growth method [Lepkovsky & Jukes, 1936; Jukes & Lepkovsky, 1936] may prove a rival to the rat growth method.

We admit that the methods described in this paper for the determination of yeast eluate factor and yeast filtrate factor may be capable of improvement. For example, it might be found advantageous, especially in the case of the determination of yeast eluate factor, to extend the "running out period" by 1 week or more, in order further to reduce the growth rate immediately prior to adding the test material to the diet. The tests carried out as described above have, however, proved very satisfactory for the rapid testing of vitamin fractions obtained in experiments on the purification of the factors.

SUMMARY

1. Rat growth methods are described for the determination of yeast eluate factor (vitamin B_6 of György; factor 1 of Lepkovsky) and yeast filtrate factor (factor 2 of Lepkovsky).

2. In both methods graded growth responses to graded doses of the factors were observed, and although the suitability of these tests for the accurate biological determination of these factors has not been worked out, the probability is that these methods, perhaps with some modification, will prove satisfactory.

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CCLXXXV. THE WATER-SOLUBLE B-VITAMINS OTHER THAN ANEURIN (VITAMIN B₁), RIBOFLAVIN AND NICOTINIC ACID REQUIRED BY THE PIG

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FROM the results of previous work [Birch *et al.* 1937; Chick *et al.* 1938, 1, 2] it was concluded that young pigs could be reared successfully on a diet of maize and purified casein with the addition of a suitable salt mixture, provided that nicotinic acid were also given. Similar experiments made on rats showed that the addition of nicotinic acid was not required for this species [Chick *et al.* 1938, 2].

The fact that an extraneous source of nicotinic acid or its amide does not appear to be required by the rat is responsible for the misleading results of researches into the aetiology of pellagra in which this animal was used. The dog, the pig and the monkey appear to resemble more closely the human being in their requirement of nicotinic acid in their food and consequently in their failure to maintain health on diets consisting too largely of maize.

The vitamin requirements of different species of animals being so various, it seemed worth while to ascertain what other heat-stable, water-soluble, accessory factors in addition to nicotinic acid and riboflavin, are required by pigs. Whatever they may be, it is clear from our previous observations [Chick *et al.* 1938, 1, 2] that they are contained in whole maize in sufficient amount when it comprises 80 % of the diet.

Edgar & Macrae [1937] have concluded that at least two other " B₂-vitamins " are necessary for rats. One of these they call "filtrate", the other "eluate" factor. Fractions containing these two factors were originally derived from yeast but Edgar *et al.* [1938, 2] have also separated similar fractions, using somewhat modified methods, from a water-acetone extract of liver.

In the present enquiry we are dealing with "filtrate fraction" and "eluate fraction" as described by Edgar and Macrae and prepared by their methods. Fractions derived by similar methods from yeast, liver and rice polishings have been prepared by Lepkovsky *et al.* [1936] and by other workers. The relation of these to those employed by us will be discussed later.

EXPERIMENTAL METHODS

It was first necessary to find a simple basal diet suitable for young pigs which would contain an adequate amount of vitamins A, D and E and of linoleic acid but which was as free as possible from water-soluble vitamins. To the basal diet a mixture containing vitamin B₁ (aneurin), riboflavin and nicotinic acid, all

given as pure chemicals, could be added as required. The following was found satisfactory:

Purified maize starch	...	...	71
Purified casein	...	...	21
Cod liver oil	...	...	2·5
Cotton seed oil	...	...	2·5
Salt mixture	...	...	3

It contained protein 18·1%, fat 6·0%, carbohydrate 64% and had a nutrient ratio of 1 to 4·2. As the pigs increased in weight the proportion of casein was gradually reduced to 15%. At first wood-charcoal was supplied as an unobjectionable form of roughage. This was found unnecessary and later discarded.

The maize starch was the purest sample obtainable and had a nitrogen content of 0·03%. It was prepared from dent white maize by the following process. The grain was steeped in a warm dilute solution of SO_2, then broken; the embryos were separated by flotation, the fibre by sieving and the protein by a process of differential sedimentation in an alkaline medium. Finally the starch deposit was washed by decantation until the nitrogen (protein) content was reduced to the required figure, after which it was filtered and dried rapidly in a current of dust-free air.

The cod liver oil was certified to contain per g. 1000 I.U. of vitamin A and 100 I.U. of vitamin D. The casein was "Glaxo ashless extracted" casein, described by the manufacturers as a casein of the "self soured" type, thoroughly washed with dilute acetic acid and afterwards extracted with hot alcohol; it contained 94% protein.

The salt mixture used was based on that recommended by Hubbell et al. [1937] who found it adequate for rats on a similar diet. It contained $CaCO_3$ 38%, bone ash 23·2%, NaCl 10·5%, KCl 27·2%, KI 0·008% and Fe_2O_3 0·84%. At first it formed 3% of the diet. Later it was realized that for rapidly growing pigs this mixture was low in phosphorus, the proportion of which in the diet, allowing for that in the casein, was less than 0·3%. The deficiency was rectified by mixing one-fifth of its weight of sodium dihydrogen phosphate (NaH_2PO_4, $2H_2O$) with that of the original salt mixture and adding 4% of the mixture to the diet. The phosphorus in the salt mixture was thus increased from 3·9 to 6·6% and that in the diet to 0·4%.

The riboflavin used was a sample of pure synthetic material kindly given by Messrs Hoffmann-La Roche, Basle, to whom we are also indebted for the specimen of nicotinic acid used.

The aneurin was pure synthetic hydrochloride provided by Prof. A. R. Todd.

The yeast was a commercial preparation of dried brewer's yeast.

Filtrate and eluate fractions prepared from liver

The laboratory preparation from yeast of the large amounts of filtrate and eluate fractions required in these experiments was not feasible and we had therefore to seek other sources. It was found by Edgar et al. [1938, 2] that fractions with similar biological properties, as tested on rats, were present in liver and were contained in the residues discarded during the commercial manufacture of some of the preparations used in the treatment of pernicious anaemia. These liver residues were kindly put at our disposal by Messrs Glaxo Laboratories, Ltd., who also prepared some of the fractions.

The liver residue available to us at the beginning of our experiment was the filtrate from the charcoal treatment of an aqueous acetone extract of liver.

Filtrate fraction was prepared from this by simple extraction of the acidulated liver residue with amyl alcohol, the filtrate fraction passing into the amyl alcohol. Eluate fraction was prepared from the material which had been extracted with amyl alcohol, by adsorption on fuller's earth and subsequent elution with barium hydroxide.

While the experiments were in progress the above liver residue became unobtainable, but two new residues were available. The first residue, that portion of an aqueous acetone extract of liver which was unextractable by phenol, was found to be a good source of eluate fraction and towards the end of our experiments we prepared an eluate fraction from this residue by adsorption on fuller's earth, elution with barium hydroxide and subsequent extraction of the eluate with amyl alcohol to remove any filtrate factor. The second residue, the filtrate from charcoal treatment of the phenol-extractable portion of the aqueous acetone extract, was rich in filtrate fraction and extraction of this residue with amyl alcohol gave the purified preparation of filtrate fraction which was used in the latter part of these experiments.

The amounts of eluate and filtrate fractions administered to the pigs were based on the requirements of rats found by Edgar & Macrae [1937] and Edgar et al. [1938, 1]. The animals in Exp. I (heavier pigs) received 100 "rat doses"[1] of filtrate fraction and 100 "rat doses" of eluate fraction daily. For the pigs in Exp. II we could not afford such a liberal allowance of eluate; they received 100 rat doses of filtrate and 50 rat doses of eluate daily. These allowances were chosen as being roughly proportional to the food intake of the pigs compared with that of the rats. At the beginning of the experiment each pig consumed about 50–100 times as much dry food as a young rat. The above allowances, however, were not increased or decreased as the pigs consumed more or less food during the course of the experiment.

The amounts given would appear to have been adequate, as 5 pigs (nos. 60, 62, 63, 65, 66) grew well when receiving both fractions. The dose of filtrate, at least, was probably not excessive, as pigs (nos. 60, 63) in which the dose of filtrate was reduced for 2 weeks showed an immediate diminution in growth, which was restored as quickly when the dosage was increased.

All the pigs received the basal diet and, except for those having yeast (see below), daily doses of aneurin 1 mg., riboflavin 2 mg. and nicotinic acid 25 mg. The doses of aneurin and riboflavin, like those of eluate and filtrate fractions, were calculated on the requirements of rats. The daily dose of 25 mg. nicotinic acid was adopted on the basis of previous experiments with pigs [Chick et al. 1938, 1] in which 60 mg. daily produced an immediate and striking effect in animals which had been depleted for 2–3 months; we decided that this dose would be excessive as a prophylactic. Sebrell et al. [1938] found 10 mg. nicotinic acid twice weekly an effective prophylactic dose to prevent black-tongue in dogs of about 6 kg. weight.

The pigs were divided into groups of 2–4 animals; one group received in addition to the above a daily dose of Edgar and Macrae's filtrate fraction, a

[1] The "rat doses" of filtrate factor and eluate factor, referred to in this paper, are equivalent to the units of these factors defined by Edgar et al. [1938, 1]. The unit of eluate factor is the potency of an amount of standard eluate fraction equivalent to 2 g. dry yeast and the unit of filtrate factor is the potency of an amount of standard filtrate fraction purified by amyl alcohol extraction, also equivalent to 2 g. dry yeast. When rats, whose growth rates are limited by deprivation of either filtrate factor or eluate factor, receive daily 1 unit of the appropriate missing factor, immediate growth responses occur of approximately 90 % of the maximum.

second group received doses of their eluate fraction, a third group received both fractions and a fourth group yeast. The growth and health of these various groups were then studied.

General management of the animals

The general management of the animals has been previously described in detail [Birch *et al.* 1937]. The pigs receiving the same diet lived together in the same stall; these stalls consisted of commodious built-in rooms with concrete flooring and attached to each was a separate open run. The animals had no bedding, but a wooden platform was provided for them to sleep on. The diet was made up in batches of 50 lb. at a time; it was merely stirred with water to a thin cream and fed cold. The appropriate amounts of aneurin, lactoflavin, nicotinic acid and the various fractions for the whole group were stirred in immediately before feeding. The animals were fed twice daily up to the limit of their appetite. If any food was left at breakfast a corresponding diminution was made in their supper, so that all was consumed.

EXPERIMENTAL RESULTS

Experiment I

The initial object of this experiment was to ascertain what would happen to young pigs fed upon a diet of starch, casein, cod liver oil and cotton seed oil supplemented with (1) dried yeast and (2) aneurin, riboflavin and nicotinic acid. The experiment was begun on 2 May 1938. A litter of 8 pigs, 12 weeks old and of weights varying between 51 and 70 lb., was divided into 2 groups of 4 each.

The first group contained the lightest pigs (nos. 53, 54, 56 and 57). They had 4% of dried yeast added to their diet and the casein and starch were correspondingly reduced. With the exception of no. 53, which died after 5 weeks and was found at autopsy to have a number of abscesses in the mesentery, these pigs made steady growth from the 2nd week onwards and gained an average of 10·9 lb. weekly. After 9 weeks the experiment was discontinued, as it was evident that 4% yeast supplied all the B-vitamin requirements over this period (see Fig. 1).

The second group (nos. 50, 51, 52 and 55) received the same diet supplemented by aneurin, riboflavin and nicotinic acid. At first the animals grew well, but in all cases growth ceased abruptly after 3–4 weeks. These pigs did not manifest any symptoms of illness but it was clear that aneurin, riboflavin and nicotinic acid did not supply all that was required. From the 7th week the eluate fraction (100 rat day doses daily) was added to the diet of nos. 50 and 55 and the filtrate fraction (100 rat day doses daily) to that of nos. 51 and 52.

Pigs receiving eluate fraction

Unfortunately no. 55 developed an acute pneumonia shortly afterwards and died. At autopsy, extensive solidification of both lungs, purulent pleurisy and pericarditis were found. This pig cannot therefore be further considered.

Pig no. 50, after a resumption of growth which lasted 5 weeks, lost its appetite, suffered intermittently from diarrhoea with the passage of blood[1] and began to lose weight rapidly. The loss of weight continued although the diarrhoea abated. It was noticed to be weak in its hindquarters and moved with

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[1] At autopsy this pig was found to have an infection of the mucous membrane of the large gut with the nematode *Oesophagostomum dentatum*.

a peculiar swaying gait. The weakness in the hindlegs increased until the animal had difficulty in maintaining equilibrium whilst eating or drinking and could not raise the hindlimbs sufficiently to scratch its body. At the end of the 14th week, that is 8 weeks after receiving the eluate fraction, the filtrate fraction was also added to its diet. A slight temporary improvement in appetite and general condition followed but the feebleness soon returned and the paresis of the hind quarters increased. Finally food was refused and the animal was killed.

Pigs receiving filtrate fraction

Pigs nos. 51 and 52 responded at once to the addition of filtrate fraction and for 1 month increased in weight at nearly the normal rate. At the end of this time growth ceased abruptly and during the next month No. 51 grew not at all and no. 52 but 7 lb. Both pigs then showed another spurt in growth which lasted for 3 weeks, during which each gained 20–25 lb. At the 10th week from the time they first received filtrate fraction growth again ceased in both of them (see Fig. 1). The first spurt of growth was understandable but the second was not. The latter coincided with the administration of a new preparation of filtrate fraction and ceased abruptly when that supply was exhausted. The most obvious interpretation would be that this particular preparation of filtrate fraction had been imperfectly fractionated, but we could not discover, by experiments upon rats, that it contained any eluate fraction. Pigs nos. 51 and 52 were occasionally observed to have epileptic fits.

Experiment II

This experiment, which was a continuation of the preceding one and over-lapped it, was begun on 30 May 1938 with 11 9-week old pigs from a litter which had just been weaned; their weights ranged from 25 to 39 lb.

Two of the lightest pigs were used to find out how much dried yeast was needed to supplement the basal casein-starch diet. From the rate at which nos. 54, 56 and 57 of Exp. I were growing we concluded that 4 % in the diet was perhaps excessive. After 3 weeks on the basal diet, the diet of nos. 59 and 67 therefore had 2 % yeast added to it. The subsequent growth was, however, very poor, and after a further 3 weeks the yeast supplement was increased to 6 %. Immediate increase in growth rate followed and the average gain in weight soon exceeded 10 lb. per week. When this occurred the amount of yeast was reduced to 4 %, and the rate of growth was maintained. We conclude, therefore, that the amount of yeast required to supply all the B-vitamins for pigs of this age, on so simple a diet as that here used, is between 2 % and 4 % and is nearer 4 %.

The remaining 9 pigs of this litter were used to ascertain the effect of giving the filtrate fraction and the eluate fraction alone and in combination. As the pigs were a month younger than those of Exp. I it was thought likely that the effects might be more striking.

For the first 3 weeks all 9 pigs received the basal diet with the additions only of aneurin, riboflavin and nicotinic acid, in the same dosage as was employed with the first litter. This procedure was adopted as a measure of economy, for we had difficulty in making enough of the eluate and filtrate fractions to keep pace with the requirements of the animals and it was thought that depletion of their reserves during this preliminary period would abbreviate the experiments. The pigs showed a slow, subnormal rate of growth.

At the end of the preliminary period 3 pigs (nos. 58, 61 and 64) received the eluate fraction in daily amounts equivalent to 50 rat day doses, 3 pigs (nos. 60,
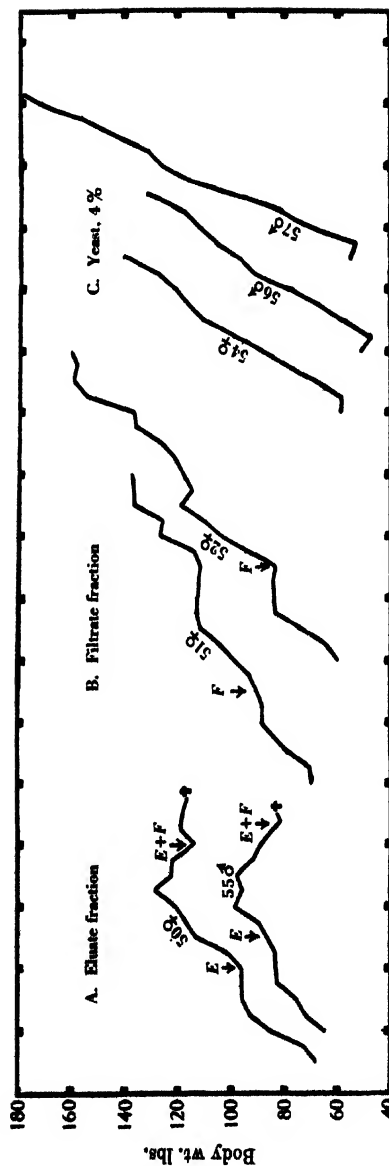
Fig. 1. Exp. I. Growth curves of young litter-mate pigs receiving a basal diet of purified casein, purified maize starch, cottonseed oil, cod liver oil and salt mixture, with daily rations of aneurin 1 mg., riboflavin 2 mg., and nicotinic acid 25 mg. Curves A, supplemented with 100 rat day doses of eluate fraction daily. Curves B, supplemented with 100 rat day doses of filtrate fraction daily. Curves C, basal diet supplemented with 4 % dried yeast. E signifies addition of eluate fraction; F signifies addition of filtrate fraction; + signifies death. Divisions on the abscissa indicate periods of 4 weeks.
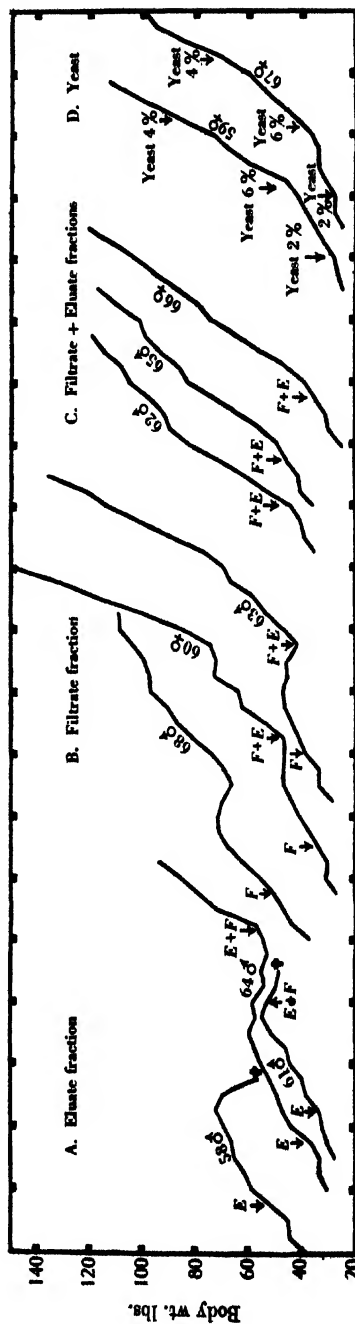
Fig. 2. Exp. II. Growth curves of young litter-mate pigs receiving a basal diet of purified casein, purified maize starch, cottonseed oil, cod liver oil and salt mixture, with daily rations of aneurin 1 mg., riboflavin 2 mg. and nicotinic acid 25 mg. Curves A, supplemented with 50 rat day doses of eluate fraction daily. Curves B, supplemented with 100 rat day doses of filtrate fraction daily. Curves C, supplemented with both fractions. Curves D, basal diet supplemented with dried yeast. E signifies addition of eluate fraction; F signifies addition of filtrate fraction; + signifies death. Divisions on the abscissa indicate periods of 4 weeks.

63 and 68) received the filtrate fraction in similar dosage and 3 pigs (nos. 62, 65 and 66) received both fractions. The growth curves of all these pigs are shown in Fig. 2.

Pigs receiving eluate fraction only

The pigs receiving eluate fraction only (nos. 58, 61 and 64) grew subnormally for 6–7 weeks, the average weight increase being about 3 lb. weekly. Afterwards appetite failed and there was an abrupt cessation of growth and a decline in weight; nos. 58 and 61 died 2–3 weeks later. Shortly before growth ceased, it was noticed that all 3 pigs had weakness of the hind quarters and sagging of their backs. These symptoms increased day by day and the gait became unusual, the hind quarters swinging as they walked. It appeared as if the back end of the pig were loosely attached to the rest of the body. The hind legs were slightly flexed at all 3 joints so that the hind portion of the body was lowered. The clumsiness of the movements of the hindquarters was most noticeable when turning and increased day by day, the condition becoming progressively worse until, after about 10 weeks from the commencement of the experiment, nos. 58 and 61 were almost completely paralysed in their hindquarters. They were unable to raise them from the ground and if lifted could not remain standing. It was a flaccid palsy and at no time spastic.

For some weeks the animals pulled themselves about with their front legs dragging the collapsed hinder end of their bodies, which were rotated laterally. At this stage the deep reflexes were absent and the pigs appeared to be insensitive over the distal half of their hindlimbs, since pricking, either superficial or deep, elicited no response. The fore quarters and head and neck were unaffected. The pigs could still eat but had to be fed by hand. No. 61 was given filtrate fraction when it was severely ill. It became brighter and for a day or two its appetite improved, but it died a few days later.

The third pig in this group, no. 64, maintained its weight rather longer. The early symptoms of damage to the nervous system occurred at about the same time as in the others but did not develop to the same extent. This animal had the same swinging gait and clumsiness of the hindquarters and often fell when turning quickly, but it could always stand or get up on its hindlegs, perhaps after one or two unsuccessful efforts. The paresis of the left hindleg was greater than that of the right and the animal would stand on 3 legs, swinging it loosely back and forward. It remained in much the same condition for a month. 13 weeks after receiving the eluate fraction it was given the filtrate fraction also, in amounts equivalent to 200 rat doses daily. Improvement in the general condition of the animal occurred immediately. Its appetite returned and during the next week it consumed 3 times as much food as before and put on 15 lb. in weight (see Fig. 2). At the same time it became much stronger and learnt to make the best of its disablement. The paresis of the left leg and clumsiness in movement, however, remained.

Pigs receiving filtrate fraction only

The 3 pigs (nos. 60, 63 and 68) which were given filtrate fraction only, remained in better condition than those having the eluate fraction. The improved rate of growth which occurred when filtrate factor was first added, after the pigs had been 3 weeks on the basal diet alone, lasted 3–4 weeks. For the next month, however, weights remained stationary. Nos. 60 and 63 were then given the eluate fraction in daily amounts equal to 50 rat doses. Their appetite immediately increased and they started growing well, their weekly increase in weight steadily increased and in 6 weeks reached 15 lb.

No. 68 continued to have filtrate factor only and from 12 August to 5 September 1938 the same unaccountable spurt in growth occurred as was shown at the same time by nos. 51 and 52 of the previous litter (see above, p. 2211). We at first suspected the particular sample of filtrate fraction, but after that sample had been replaced by another, no. 68, after a stationary period, again resumed a moderate rate of growth (Fig. 2), suggesting that our suspicions were unfounded and that intermittent growth may be characteristic of deficiency of the eluate fraction.

None of the pigs deprived of the eluate factor seemed particularly ill but they all suffered from epileptic fits. These were first observed in no. 63, 7 weeks after the commencement of the experiment, when growth had just ceased. During the next week no. 60 was seen in a fit and 3 weeks later no. 68. It is impossible to say how frequently the fits occurred as the animals were not under observation during the greater part of the 24 hr.

Only one fit was observed in nos. 60 and 63 after they were receiving the eluate fraction as well as the filtrate, and that occurred on the day following administration of the former. In the case of no. 68, which was kept without eluate for 20 weeks, the epileptic attacks became more frequent, as many as 5 being observed in 1 week and 2 on 1 day.

The pigs receiving the filtrate fraction only were also noticed to have become pale. Estimations of haemoglobin in their bloods 2–4 weeks after they had ceased to grow (8 weeks after the beginning of the experiment) showed it to be less than 60 % of the amount usually present in the blood of normally fed pigs of this age or in the blood of the pigs which were receiving yeast.

Pigs nos. 60 and 63 received the eluate fraction as well as the filtrate fraction from the 10th week onwards; the anaemia was steadily repaired and 8 weeks later their blood was nearly normal.

Details concerning the blood of all these pigs will be found on p. 2217, Table III below.

Pigs receiving both filtrate and eluate fractions

The 3 pigs (nos. 62, 65 and 66) which received both eluate and filtrate fraction at the same time as the preceding groups received one only of these fractions, remained healthy and grew steadily during the 11 weeks they were observed after these additions were made to their diets. They had no nervous symptoms and their blood remained normal. They did not, however, grow so well, although they ate as much, as those receiving yeast. Their average increase in weight per week was 7 lb. whereas that of the latter was 8·5 lb. It was also noted that their appetite diminished towards the end of the period whereas it should have become greater, having regard to their increased body weight.

We are able to compare the gain of weight per unit of food consumed by the pigs in these 2 groups over periods of 4 weeks, during which all the animals were of approximately the same weight. The pigs to which yeast was given gained 15·5 kg. and consumed 22 kg. of dry food. Those having both eluate and filtrate gained 16 kg. and consumed 29 kg. food. If 0·5 kg. of dry food per day be subtracted as the maintenance allowance for pigs of this weight—an amount representing the average amount of this diet required to maintain weight—the productivity, $\dfrac{\text{Wt. gained}}{\text{Food consumed}-\text{maintenance allowance}}$, was 1·4 for the diet with yeast and 1·0 for the diet with eluate and filtrate.

These facts suggest either that the supplements of aneurin, riboflavin, nicotinic acid, eluate fraction and filtrate fraction were not given in adequate amount,

or that, when combined, they do not possess quite all the nutritive virtues of whole yeast, a conclusion similar to that reached by Edgar et al. [1938, 3] from their experiments with rats.

HAEMATOLOGICAL OBSERVATIONS

Anaemia from lack of mixtures of B-vitamins has been recorded in rats by Damianovich et al. [1923], Whitehead & Barlow [1929], Sure et al. [1931] and by György et al. [1937]; in pigeons by Barlow [1927] and by Hogan et al. [1937]; in dogs by Rhoads & Miller [1933], Spies & Dowling [1935] and by Fouts et al. [1938]; in monkeys by Day et al. [1935], Wills & Stewart [1935] and by Wills & Evans [1938]; in pigs by Miller & Rhoads [1935], Birch et al. [1937] and by Wintrobe et al. [1938].

The observations of Miller & Rhoads [1935] and of Wintrobe et al. [1938] are particularly pertinent to the present investigations because they concerned pigs, and those of Fouts et al. [1938] because they cured the anaemia with a fraction from rice polishings containing the "rat anti-dermatitis factor" (vitamin B_6). As will be seen below (p. 2221), Edgar and Macrae's eluate fraction which prevented or cured the microcytic anaemia we encountered in our pigs also contains vitamin B_6.

Systematic observations on the blood of the pigs were not carried out from the beginning of our experiments but as after 8-10 weeks on the deficient diets all the pigs were observed to be paler, determinations of haemoglobin and examinations of blood films were undertaken. These showed that a considerable degree of anaemia existed both in the animals deprived of eluate fraction and in those deprived of filtrate fraction and that the anaemia of the former was associated with the presence of large numbers of small corpuscles, $3-4\mu$ in diameter (microcytic). Henceforth, a more complete examination of their bloods was made from time to time. The observations are set out below in tabular form (see Tables I–III). Incomplete as they are, they permit of some definite conclusions concerning the effect on the blood picture of deprivation of Edgar and Macrae's filtrate and eluate fractions.

The blood was drawn from a vein either of the ear or of the tip of the tail. The haemoglobin determinations were made with a Sahli haemometer and checked with a Haldane CO haemoglobinometer. Fresh standards were prepared of such strength that the 100 mark on the tubes corresponded to 18·5 vol. O_2 or 13·8 g. haemoglobin per 100 ml. blood. The error of the pipette was ascertained by drawing human blood to the 20 c.mm. mark, allowing it to clot, weighing the pipette filled with blood and subtracting the weight of the pipette. The specific gravity of the blood was assumed to be 1·060. The graduated tubes were calibrated by adding approximately 1 and 2 g. water, weighing them and noting the levels in the tubes.

The corpuscular volume was ascertained by centrifuging bloods containing 0·2 % potassium oxalate in tubes of 1 mm. bore and 10 cm. length for half an hour at 4000 r.p.m. The proportion of the length of the column of corpuscles to that of the blood was multiplied by 1·1 to compensate for the shrinkage of the corpuscles due to this strength of oxalate [Wintrobe, 1931].

The mean corpuscular vol. in μ^3

$$= \frac{\text{Vol. corps.}}{\text{No. of corps.}} \text{ per } \mu l. \times 10^9.$$

Dr Lucy Wills very kindly measured the red corpuscles and plotted Price Jones curves from films taken from pigs nos. 61 and 64 which had been deprived

of filtrate fraction and from pigs nos. 63 and 68 which had been deprived of eluate fraction, and compared them with films taken from normal pigs of about the same age. The mean diameter, standard deviation and coefficient of variability arrived at from this laborious procedure, for which we cannot be too grateful, were:

No. pig	Mean diameter μ	Standard deviation	Coefficient of variability
Normal	5·87	0·48	8·2
61	5·83	0·69	11·8
64	6·25	0·49	7·9
63	5·64	0·69	11·8
68 (19. viii. 38)	5·16	0·98	18·9
68 (13. ix. 38)	5·54	0·84	15·9

The films supplied were poor ones, which perhaps accounts in some measure for the high standard deviation from the mean diameter. The changes in mean diameter are consistent with the results obtained by the haematocrit method, which by itself gives no indication of the variability in size of the corpuscles.

The data obtained from the examination of the blood of pigs nos. 59 and 67 will serve as a standard of reference (see Table I). These pigs taken from the same litter had been fed for 2 months on the basal diet supplemented by yeast: they were growing rapidly and were in excellent condition. The figures are close to some recently obtained by Mr Parry (personal communication) at the Institute of Animal Pathology, Cambridge, for healthy pigs somewhat younger than the present ones fed on an ordinary good diet. Mr Parry's data were obtained by methods precisely similar to ours. They also correspond closely with the averages of those found by Miller & Rhoads [1935] for their 15 young pigs before they received a deficient diet. We therefore assume that they may be taken as fair average values for normal pigs.

Table I. *Blood examination of normal pigs*

No. pig	R.B.C. millions per c.mm.	Hb, g. per 100 ml. blood	Corpuscular vol. %	Mean corpuscular vol. μ^3	Hb. g. per 100 ml. corpuscles	Remarks
I. Pigs nos. 59 and 67 after 10 weeks on basal diet supplemented by yeast; age 5 months; wts. 108 and 95 lb. respectively						
59	7·8	12·9	43	55	30	Films show little scatter in the size of cells; most appear to be about 6μ in diameter
67	8·1	12·4	44	54	28	
II. Pigs nos. 62, 65 and 66, after 3 weeks on basal diet and 10 weeks on basal diet supplemented by both eluate and filtrate fractions; age 5 months; wts. 115, 109 and 112 lb. respectively						
62	7·5	11·8	40	53	29	Most cells appear to be around 6μ in diameter
65	8·1	12·4	42	52	29	
66	8·0	12·9	45	56	28	

The effect of deprivation of filtrate fraction was to produce a moderate degree of anaemia in which both the number of blood corpuscles and the concentration of haemoglobin were reduced to about 2/3 of their normal amounts. The size of the corpuscles underwent little change. This type of anaemia is similar to that observed by Wintrobe *et al.* [1938] in pigs receiving minimal amounts of yeast. On receiving filtrate fraction, the composition of the blood of pig no. 64 (the only surviving pig in this group) showed little improvement after

Table II. *Effect on blood of deprivation of filtrate fraction*

Pigs nos. 58, 61 and 64. At the date (15. viii. 38) of the first observation they were 3 months old and had received the basal diet for 3 weeks, followed by 8 weeks on the same diet supplemented by eluate fraction; wts. 60, 50 and 60 lb., respectively. From 23. ix. 38 onwards no. 64 received filtrate fraction also.

No.	Date	R.B.C. millions per c.mm.	Hb, g. per 100 ml. blood	Corpuscular vol. %	Mean corpuscular vol. μ^3	Hb, g. per 100 ml. corpuscles	Remarks
58	15. viii. 38	—	7·9	–	—	—	Paralysed, losing wt.; died 17. viii. 38
61	15. viii. 38	—·	7·0	- -	—	—	Paralysed, losing wt.; died 25. viii. 38
64	15. viii. 38	—	9·6	·—	—	·—	Partial paralysis; wt. stationary
64	13. ix. 38	6·3	8·5	30	48	28	Films show abnormal scatter, mean diameter about 6μ
64	21. ix. 38	5·6	7·7	29	52	26	—
64	1. x. 38	5·4	6·9	26	48	26·5	Filtrate fraction also given from 23. ix. 38
64	11. x. 38	5·9	7·6	30	51	25	—

Table III. *Effect on blood of deprivation of eluate fraction*

Pigs nos. 60, 63, 68. At the date of the first observation (25. vii. 38) they were 4 months old and had received the basal diet for 3 weeks, followed by 5 weeks on the same diet supplemented by filtrate fraction only; wts. 47, 46 and 72 lb., respectively, and weight stationary. From 8. viii. 38 onwards nos. 60 and 63 were given eluate fraction also.

No.	Date	R.B.C. millions per c.mm.	Hb, g. per 100 ml. blood	Corpuscular vol. %	Mean corpuscular vol. μ^3	Hb, g. per 100 ml. corpuscles	Remarks
68	25. vii. 38	—·	7·9	—	—	·—	—
68	19. viii. 38	6·5	6·1	24	37	25	Numbers of small corpuscles of about 4μ diam.
68	13. ix. 38	7·0	5·8	24	34	24	-
68	21. ix. 38	7·1	4·2	23	32	17·5	—
68	11. x. 38	11·3	6·3	27	24	23·5	Numbers of small cells much increased
60	25. vii. 38	—	6·8	—	—	- -	Anisocytosis: large numbers of small corpuscles of about 4μ diam.
63	25. vii. 38	—	7·1	—	- -	—	
	From 8. viii. 38 nos. 60 and 63 received eluate also						
60	19. viii. 38	7·8	6·7	28	36	24	—
63	19. viii. 38	7·1	7·2	26	36	27	Still numbers of small corpuscles of about 4μ diam.
60	13. ix. 38	—	8·7	—	—	—	—
63	13. ix. 38	—	8·9	—	—	—	—
60	21. ix. 38	—	9·8	—	—	—	—
63	21. ix. 38	—	9·6	—	—	—	—
60	5. x. 38	8·8	11·7	37	42	31	Numbers of small corpuscles diminished
63	5. x. 38	8·3	12·0	33	40	36	—
60	15. x. 38	8·4	12·1	40	47	30	Few small corpuscles seen
63	15. x. 38	7·9	11·8	37	42	32	—

3 weeks, but this does not necessarily mean that its blood-forming tissue has not responded to the addition. Immediately after it received the filtrate fraction this pig started to grow at a great rate and added 25 lb. (45 %) to its body weight during the first 18 days and presumably the blood volume was also considerably increased (see Table II).

The effect of deprivation of eluate fraction was at first to cause a small decrease in the number of blood corpuscles and a considerable decrease in the size. In the case of pig no. 68, in which the deprivation was continued longest, the number of corpuscles increased later (11. x. 38) to about 50 % in excess of the usual number and their mean volume was reduced to about one half. The large proportion of small corpuscles ranging about 4μ in diameter was at once obvious on inspecting a dried film or when counting them in the haemocytometer. The amount of haemoglobin per ml. blood was half, and per unit of corpuscular volume three-quarters, of the normal, the result being a microcytic hypochromic anaemia. In those pigs, nos. 60 and 63, in which after 8 weeks the deprivation of eluate fraction was made good, the small corpuscles disappeared from the blood, slowly at first and then steadily, until after 7 weeks their size was nearly normal. At the same time the amount of haemoglobin increased and approached normality (see Table III).

Here again the initial slowness of recovery in the blood picture must again be considered in the light of the concomitant sudden increase in growth rate, and presumably in blood volume, brought about by restoration of the missing factor in the diet.

Comparison of our results with those of others leads us to conclude that there is more than one substance in the vitamin B_2 complex, the lack of which embarrasses the blood-forming tissues.

The anaemia observed by Wintrobe et al. [1938] in pigs fed on a synthetic diet not unlike that used by us, to which diminishing quantities of yeast were added as the experiment proceeded, was of the simple type which occurred in pigs nos. 58, 61 and 64 (see p. 2217, Table II above). These were the animals from which filtrate fraction was withheld. The number of red blood corpuscles and the amount of haemoglobin in their blood fell together without any obvious change in the dimensions of the red cells.

The anaemia in dogs suffering from black-tongue, produced by feeding them on Goldberger's pellagra-producing diet, was found by Rhoads & Miller [1933] to be of the macrocytic type. Later, when Miller & Rhoads [1935] turned their attention to pigs on a similar diet, they found the same evidence of increased activity of the bone marrow but the anaemia was microcytic in some animals and macrocytic in others.

Wills & Stewart [1935] produced a profound macrocytic anaemia by long-continued feeding of monkeys on polished rice, margarine, white bread and cod liver oil. Some success was obtained by treating the monkeys with Edgar and Macrae's filtrate fraction [Wills & Evans, 1938]. As it is the eluate fraction which prevents or cures the microcytic anaemia we have encountered, it is difficult to see the connexion between their experiments and ours. Nor is the precise relationship obvious between our observations and those of György et al. [1937] on aplastic anaemia in rats receiving a synthetic diet, beyond the fact that the anaemia was cured in both instances by an "impure preparation of vitamin B_6". Purified preparations of vitamin B_6, whilst curing the dermatitis characteristic of deficiency of this vitamin in rats, had no effect on the anaemia. In a later paper György [1938] concluded that the anti-anaemic substance was nicotinic acid. The anaemia in our pigs nos. 60, 63 and 68 deprived of eluate fraction,

was not aplastic in type and as it developed in spite of a daily ration of nicotinic acid, there does not appear to be much correspondence between these observations on rats and our own.

The recent observation of Fouts et al. [1938] that hypochromic microcytic anaemia, accompanied by convulsive fits, occurred in puppies maintained on a synthetic diet, do, however, afford an almost complete parallel with some of our experiments on pigs. Their basal diet included vitamins A and D, vitamin B_1, either as crystalline material or as purified concentrate, and riboflavin. The remaining constituents of the vitamin B_2 complex were provided as (1) a liver extract which contained the chick anti-dermatitis filtrate factor [Elvehjem & Koehn, 1935; Lepkovsky & Jukes, 1935; 1936] and nicotinic acid, and (2) an extract of rice polishings containing the "rat anti-dermatitis factor" (vitamin B_6). The activities of their preparations were controlled by experiments on rats and chickens. Four puppies which received the basal diet and liver extract only (Lepkovsky's filtrate factor + nicotinic acid) developed a severe anaemia and exhibited convulsive fits. The anaemia was cured when extract of rice polishings was added to the diet. Preparations (1) and (2) above correspond broadly to our "filtrate" and "eluate" fractions, respectively, except that in our preparations from yeast or liver the nicotinic acid or amide originally present in these sources was contained in the eluate fraction, being adsorbed together with it on fuller's earth at pH 1·3 and subsequently eluted, whereas in the preparation used by Fouts and his colleagues nicotinic acid was present in the filtrate after adsorption of their liver extract with Lloyd's reagent (see also below, p. 2222).

The results of the experiments of Hogan et al. [1937] are also difficult to bring into line, partly because no details are given. Pigeons were fed on a diet of casein, sucrose, cellulose and cod liver oil and, when polyneuritis had developed a concentrated preparation of vitamin B_1 was given. This cured the polyneuritis but about 2 months later the birds became profoundly anaemic. Neither riboflavin nor "anti-dermatitis concentrate" (?chick anti-dermatitis factor) was curative.

The anaemia occurring in pigs after gastrectomy, which has been studied by Bence [1933; 1934; 1936] and by Petri et al. [1937], should perhaps be mentioned, as this may be partly due to "conditioned deficiency", although the former author adduces evidence to show that it is caused by deprivation of Castle's intrinsic factor, which is manufactured in the mucous membrane of the normal stomach. According to Petri et al. [1937] the pigs manifested symptoms suggestive of pellagra and Bence's interpretation is not to be regarded as sufficient. An interesting pathological point is that, for the first year after the operation, the anaemia was of the hypochromic microcytic type associated with hyperplasia of the bone marrow. Bence reports that it subsequently became macrocytic and hyperchromic. The pigs studied by Petri and his colleagues were not under observation long enough for this result to be confirmed.

The confusion indicated in the précis of literature given above will not be cleared up until it is possible to employ pure substances in nutritive experiments in which one deficiency only is present. Even then it will probably be found that results obtained with one species of animal cannot necessarily be applied to another.

Nervous symptoms in pigs deprived of eluate factor and of filtrate factor

All the pigs in these groups had nervous manifestations; those deprived of eluate fraction had fits and those deprived of filtrate fraction became paralysed.

Altogether, some 30 fits were recorded and it is probable that more occurred when nobody was there to observe them. Their severity and frequency increased with the length of time the animals had been deprived of eluate fraction. They were typical epileptic fits as seen in the human being. The pig ran round in apparent terror, screamed (epileptic cry) and dropped as if shot, making no effort to guard the fall; tonic spasm followed during which the legs were extended. The pig became deathly white. This first stage was succeeded by a clonic stage, during which the limbs were jerked about violently; there was grinding of teeth and sometimes discharge of urine. The comatose stage followed; respiration was at first deep and stertorous, gradually becoming shallow. The pig looked as if dead but in a few minutes the colour improved, consciousness was recovered and the animal got up languidly and resumed his previous occupation, e.g. feeding. The duration of the fits was from a few minutes to a quarter of an hour. In the intervening periods the pigs appeared to be quite well. Minor epileptic manifestations were also observed in which the animals appeared dazed, butted into obstacles, tottered about without always actually falling and then rapidly regained consciousness. After the eluate fraction was added to pigs nos. 60 and 63 the epileptic seizures ceased.

In the experiments on puppies reported by Fouts *et al.* [1938] convulsions were associated with deprivation of a filtrate fraction somewhat similar to our own, prepared from liver; some of the puppies died during the fits. In experiments of earlier workers on dogs deprived of B-vitamins, convulsions were observed towards the termination of their lives, but as far as we know the only record of epilepsy being caused by a nutritive deficiency is one by Sheehy & Senior [1930], in a group of pigs deprived of vitamin D and shielded from ultraviolet rays by glass. The fits occurred from the seventh week of the experiment onwards and three of the animals died from them.

The paralytic symptoms which developed in the pigs deprived of filtrate fraction are described above (p. 2213) in the history of the animals in this group. It was a flaccid paraplegia confined to the hindquarters and accompanied by some disturbance of sensation. Once developed it was not cured by giving filtrate fraction (see pig no. 64).

Posterior paralysis of swine has been not infrequently encountered in young pigs in the United States. A good description of the disease was given by Wehrbein [1916] who found demyelination in the spinal cord and peripheral nerves. The symptoms described correspond closely with those observed in pigs nos. 58, 61, 64. Sporadic cases of posterior paralysis in pigs are also recorded by Doyle [1937]. Some of these occurred under conditions which rendered it improbable that the cause was nutritional in origin and Doyle therefore suggested that there was an infective form of the disease. The discovery of some scattered areas of small cell infiltration in the nerves and spinal cord supported this view.

Paraplegias have been reported in swine as the result of an inadequate supply of vitamin A by Hughes *et al.* [1928], Dunlop [1934], Eveleth & Biester [1937] and Foot *et al.* [1938]. The nervous symptoms observed by Dunlop were uncommonly like those seen by us, but they took a much longer time to develop. Deficiency of vitamin A can hardly have been responsible for the paralysis of our pigs which were deprived of filtrate fraction. Each pig received daily 25 g. or more of a reliable cod liver oil certified to contain more than 1000 I.U. vitamin A per g. The requirements of the pig for this vitamin were carefully determined by Dunlop [1935] who concluded that 6000 I.U. daily affords a 50% margin for safety.

The recently published observations on pigs of Wintrobe *et al.* [1938] have a close bearing upon our own. The basal diet, consisting of purified casein, butter,

sucrose, cod liver oil and salts, was similar to that used by us. At first, 3 g. yeast per kg. body-weight were given daily, this dose being subsequently gradually reduced to 0·1 g. per kg. As the yeast was reduced, crystalline aneurin and riboflavin were given. After about 200 days the animals manifested nervous symptoms. These consisted of progressive ataxia, with some loss of deep and superficial sensation but without much motor weakness. Histologically there was severe degeneration of the posterior columns of the spinal cord, the dorsal ganglion cells and the peripheral nerves.

From a scrutiny of the diet it would appear that the pigs in these experiments of Wintrobe and his colleagues were gradually deprived of nicotinic acid and of the vitamins contained in our eluate and filtrate fractions. The symptoms were more of the sensory type than in our pigs, but further comment is deferred until the nervous systems of our animals have been examined by appropriate histological methods. Until that has been done we also postpone consideration of many interesting papers on the nervous lesions produced in other animals by feeding on diets deficient in some or all of what is conveniently called the vitamin B_2 complex.

Comparison of our eluate and filtrate fractions with those of other investigators

The fractions containing water-soluble B-vitamins other than aneurin and riboflavin which have been described by various investigators have mostly been prepared from aqueous extracts of liver, yeast and rice polishings by treatment with fuller's earth, but the conditions under which the adsorptions have been carried out have varied.

It is unlikely that the extracts of liver, yeast and rice polishings from which these fractions have been separated were nutritionally equivalent and it is quite possible, even should they be so, that the individual factors were present in the various extracts in different chemical combinations. The amount and composition of other materials present differ and affect the results of the adsorption processes. Further, fuller's earth is by no means a standard product, the adsorptive properties of different samples varying considerably. It is therefore not surprising to find that after fractionation of extracts of liver, yeast and rice polishings in various laboratories with the use of fuller's earth, the different essential nutrients are not always found in the corresponding fractions. For example, in the case of yeast, the fuller's earth adsorbate contains the greater part of the nicotinamide or other active pyridine base, as has been demonstrated in experiments on dogs and pigs, while in the case of some extracts of liver and of rice polishings these compounds have been found present in the fuller's earth filtrates. Similarly, filtrates obtained after treatment of liver extracts with fuller's earth are by no means nutritionally equivalent to those obtained by exactly similar methods from yeast when tested on the rat [Edgar et al. 1938, 2].

Comparison of the liver fractions used in this work with fractions prepared by others is therefore very difficult. When tested on rats, they had the same action as the filtrate and eluate fractions prepared from yeast by means of fuller's earth [Edgar et al. 1938, 2].

The eluate fraction certainly contained vitamin B_6, the rat anti-dermatitis factor of György [1935]. We found that the crystalline factor 1 of Lepkovsky [1938], which is identical with vitamin B_6, completely replaced our eluate fraction in the diet of the rat [unpublished results]. The rice polishings eluate used in the experiments on dogs by Fouts et al. [1938], in which a nutritional anaemia similar to that now observed in pigs was noted, would be similar to our liver eluate factor, in so far as they both contained vitamin B_6. It is

probable that our eluate fraction also contained nicotinamide [cf. Dann & Subbarow, 1938]. Since, however, our pigs received nicotinic acid in their basal diet, the possibility that nicotinamide was the essential nutrient supplied by the eluate fraction is excluded. Pigs deprived of eluate fraction were certainly deprived of vitamin B_6 but, until the experiments can be repeated with the use of crystalline vitamin B_6, the question whether all the symptoms observed are due to a deficiency of this vitamin must remain unanswered.

Our filtrate fraction apparently contained the nutrient required for growth of rats named factor 2 by Lepkovsky et al. [1936]. The growth-promoting action of their factor 2 for rats was very similar to that possessed by our liver filtrate fraction.

Lepkovsky & Jukes [1936] first gave the name "filtrate factor" to the substance obtained after treatment with fuller's earth of an extract of beef liver. This substance had a specific effect in the prevention and cure of the dermatitis described by Elvehjem & Koehn [1935] which occurs in chicks receiving a diet of yellow maize, wheat middlings and casein heated for 144 hr. at 100°. Lepkovsky & Jukes showed that their filtrate factor withstood autoclaving at pH 5·0, was not precipitated by lead acetate at pH 6·6 or by barium hydroxide, was not adsorbed on norite charcoal or lead sulphide and was extracted by amyl alcohol. Although in many respects our filtrate factor possessed similar properties to these, it is uncertain whether the chick "anti-dermatitis factor" which was also present in Lepkovsky's factor 2 and is possibly identical with the rat growth filtrate factor of Edgar and Macrae, would be contained in all the preparations of "filtrate fraction" used by us. From the properties described for this chick factor, the earlier batches used by us ought to have contained it, but the later batches made from phenol extracts would only have contained it if it is extractable by phenol. It is most improbable that our filtrate fraction would contain nicotinamide or other pyridine bases, since one step in its preparation was extraction from strong acid solution by amyl alcohol. The liver filtrate fractions used by Fouts et al. [1938] would probably contain all factors present in our filtrate fraction, and nicotinamide in addition. The relation of our fractions to the Elvehjem factor W [Elvehjem et al. 1936; Frost & Elvehjem, 1937] is obscure.

As was the case with our eluate fraction it is impossible to tell at present whether the vitamins in our filtrate fraction which are essential for the rat and the pig, respectively, are identical.

SUMMARY

1. Pigs can be reared on a synthetic diet containing purified casein, purified starch, cottonseed oil, cod liver oil and a suitable salt mixture, and optimum growth can be obtained, when 4 %, but not 2 %, dried yeast is added.

2. When the above diet was supplemented by aneurin, riboflavin and nicotinic acid, growth ceased after 3–5 weeks according to the age and reserves of the animals at the beginning of the experiment.

3. If to the diet supplemented as described under 2 was added either the eluate fraction of Edgar and Macrae or their filtrate fraction, prepared from liver, growth proceeded at about one-third the normal rate, but was checked or arrested after 4–6 weeks.

4. When the eluate fraction was added to the diet of pigs which had previously received the filtrate fraction only and whose growth was arrested, there was an immediate and continuous gain in weight.

5. Addition of filtrate fraction to the diet of those pigs which had previously received only eluate fraction was followed by immediate improvement in appetite and rapid increase in weight in the one case in which paralysis was slight and the animal was not too ill to respond. The other 3 animals died in a few days after the filtrate was administered.

6. When, after about 3 weeks on the basal diet supplemented as described under 2, both eluate and filtrate fractions were given, there was an immediate response in food intake and good growth was observed for about 5 weeks, after which there was no further increase in appetite and the rate of gain in weight slackened accordingly. It is therefore probable that yeast contains some unidentified essential nutrient for the pig in addition to aneurin, nicotinic acid, riboflavin and what may be contained in the eluate and filtrate fractions.

7. Pigs maintained on the basal diet supplemented by eluate only, developed a flaccid palsy of the hindquarters.

8. Pigs receiving supplements of filtrate only, developed a microcytic anaemia and had typical epileptic fits. On receiving supplements of the eluate the blood returned to normal and the fits ceased.

9. In addition to aneurin, nicotinic acid and probably riboflavin, at least two further water-soluble vitamins are necessary for the nutrition of the pig. These vitamins are contained in the filtrate and eluate fractions of Edgar and Macrae; whether the whole activity of the eluate fraction is to be accounted for by the vitamin B_6 which it contains, cannot be decided until this vitamin is available in the pure state in sufficient quantity to be tested on pigs.

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Note added 7 December 1938. Since the above paper was sent to press we have received the report of work by Hughes [1938, *Hilgardia*, 11, 595], on the requirements of the pig for different members of the vitamin B complex. Although his basal diet and supplements were not as highly purified as those used by us, his observations were made on more animals, 83 young pigs in all; the results are in general agreement with our own. Vitamin B_1, riboflavin given as whey adsorbate, nicotinic acid and "rice bran filtrate" (Lepkovsky's factor 2) were all needed to supplement a basal diet of polished rice, purified casein, salt mixture and cod liver oil. The whey adsorbate, given as source of riboflavin, doubtless contained our "eluate factor". The growth observed with the above supplements was superior to that occurring when 3 % yeast was added to the basal diet. This is in agreement with our observations indicating 4 % yeast as the minimum required to ensure an adequate supply of all the B-vitamins.

CCLXXXVI. THE WATER-SOLUBLE B-VITAMINS

XII. THE PREPARATION FROM LIVER OF FRACTIONS CONTAINING THE SAME DIETARY ESSENTIAL FACTORS FOR THE RAT AS YEAST ELUATE FRACTION AND YEAST FILTRATE FRACTION

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IN this and certain other laboratories yeast has been employed as a source of the B-vitamins, whilst some workers have favoured the use of liver. Similar experimental methods have been used for the fractionation of liver and yeast extracts, and of these adsorption with fuller's earth has been most generally employed. We have obtained by this means two fractions from autoclaved yeast extracts containing distinct rat dietary essentials. These we have called yeast eluate factor and yeast filtrate factor [Edgar & Macrae, 1937].

The identity of yeast eluate factor, vitamin B_6 [György, 1935] and factor 1 [Lepkovsky *et al.* 1936] is established [Edgar, El Sadr & Macrae, unpublished]; the relationship of the yeast filtrate factor to similar factors described by other workers is discussed in the preceding paper [Edgar *et al.* 1938].

Fractionation of liver by the methods we used to separate the factors present in yeast extracts did not yield fractions with biological properties corresponding to those of the yeast fractions. However, by other methods we have obtained fractions from liver extracts biologically similar to the yeast eluate and filtrate fractions. The present paper describes these experiments.

EXPERIMENTAL

In general the rat growth method used in the testing for yeast eluate and yeast filtrate factors has been employed [Edgar *et al.* 1938]. The preliminary preparation of the rats was carried out as described, the young animals receiving for the first week after weaning the same basal diet and daily supplements of 0·08 ml. of cod liver oil and 10 μg. of aneurin. Thereafter, the experimental methods were varied to some extent, as is described in the separate experiments.

Yeast eluate fraction and yeast filtrate fraction were prepared by the methods described in the previous paper [Edgar *et al.* 1938]. All the liver fractions were prepared from liver residues obtained in the manufacture of the pernicious anaemia factor [Laland & Klem, 1936]; these were kindly supplied to us by Messrs Glaxo Laboratories. Three residues have been investigated.

Liver residue I. This residue was the filtrate resulting when a liver extract, obtained by extraction of liver with aqueous acetone and subsequent removal of the acetone, was treated with charcoal. Experiments with rats showed that this residue probably contained all of the factors of the vitamin B_2 complex with the exception of riboflavin, since rats receiving the basal diet supplemented by cod

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liver oil, aneurin, riboflavin and this liver residue grew as well as did rats receiving that diet with whole yeast or liver extract as the source of the B_2-vitamins.

Liver residue II. The liver extract made by aqueous acetone extraction of liver, freed from acetone and reduced to small volume was extracted with phenol. The fraction insoluble in phenol was liver residue II. This liver fraction was also rich in members of the vitamin B_2 complex, although it contained less of the liver factor corresponding to yeast filtrate factor than did liver residue I.

Liver residue III. The aqueous layer, obtained when the above phenol extract was shaken with water and ether, was treated with charcoal. Liver residue III was the filtrate from the charcoal. This residue contained considerable amounts of the rat dietary factor corresponding to the yeast filtrate factor and only traces of other factors.

Liver residue I has been mainly used in this investigation.

Fractionation of liver residue I with fuller's earth

The liver fraction (1 l.; 1 ml. = 5 g. fresh liver) was adjusted to pH 1·2 by the addition of H_2SO_4, and 50 g. fuller's earth ("specially selected, activated", Fuller's Earth Union) was added. After stirring at intervals for 30 min. the adsorbate was removed by filtration. The adsorption was repeated, and the filtrate, after treatment with $Ba(OH)_2$ to remove H_2SO_4, was ready for administration to rats.

The first adsorbate was washed twice with 500 ml. of $N/10$ HCl and suspended in 700 ml. of 2% $Ba(OH)_2$. After several hours at 0°, the eluate was removed by filtration, and the adsorbate was again eluted with 500 ml. of 2% $Ba(OH)_2$. The combined eluates, freed from Ba with H_2SO_4, were adjusted to pH 8 and treated with an excess of basic lead acetate (8 g. in 40 ml. of H_2O). The resulting precipitate was filtered off and the excess lead removed from the filtrate with H_2S. The PbS was filtered off and the filtrate reduced *in vacuo* to 250 ml. (1 ml. = 20 g. fresh liver).

Young rats, prepared as described, having received the basal diet and the cod liver oil and aneurin supplements for 1 week, were each given additional daily supplements of 50 μg. of riboflavin and either 1 ml. of the liver fuller's earth filtrate (= 5 g. fresh liver) or 1 ml. of liver fuller's earth eluate (= 20 g. fresh liver).

The animals receiving the liver fuller's earth filtrate gained in weight at an average rate of 30 g. weekly for 3 weeks (Table I). This growth rate is nearly double that obtained when rats receive the diet supplemented by yeast fuller's

Table I. *Effect of fractionation of liver residue I with fuller's earth*

Each rat (male) received daily 10–15 μg. aneurin and 50 μg. riboflavin

No. of rats	Daily supplement given during period of 3 weeks	Av. weekly wt. increase of group during 3-week period (g.)	Additional daily supplement given during 4th week	Av. weekly wt. increase of group during 4th week (g.)
2	Fuller's earth filtrate from liver residue I (= 5 g. fresh liver)	35, 29, 27	Fuller's earth eluate from liver residue I (= 20 g. fresh liver)	37
2	Fuller's earth filtrate from liver residue I (= 5 g. fresh liver)	33, 28, 29	None	26
4	Fuller's earth eluate from liver residue I (= 20 g. fresh liver)	22, 15, 10	Yeast fuller's earth filtrate not purified by amyl alcohol extraction (= 1 g. dry yeast)	25

earth filtrate (see Table II), indicating that the liver filtrate contains other dietary essentials for the rat in addition to those present in yeast filtrate fraction. The addition of liver fuller's earth eluate fraction to the diet after 3 weeks did, however, cause an increase in the growth rate, showing that the liver fuller's earth filtrate was deficient in a growth factor for rats which was contained in the liver fuller's earth eluate fraction.

The rats which received the liver fuller's earth eluate fraction from the end of the first week after weaning gained less in body weight than those receiving filtrate. Here again, however, the growth rate was somewhat in excess of that occurring after administration of yeast eluate (see Table II), suggesting the presence of other dietary essentials in this liver fraction. The addition of yeast filtrate fraction to the diet caused a striking increase in the growth rate, which proved that the dietary essential contained in the yeast filtrate fraction was a limiting factor in this liver fuller's earth eluate fraction.

The above procedure therefore, which is essentially the same as that which was employed in the separation from yeast of the yeast eluate and yeast filtrate factors, did not yield fractions from liver with the same biological properties as those from yeast.

Preparation from liver residue I of fractions biologically similar to the yeast filtrate and yeast eluate fractions

Since fuller's earth fractionation of liver extract did not yield fractions corresponding to the yeast fractions, other methods of separation had to be employed. The liver preparations described below with activities similar to those of the yeast filtrate and eluate fractions we have named liver filtrate fraction and liver eluate fraction, respectively.

Liver filtrate fraction. Liver residue I (500 ml.; 1 ml. = 10 g. fresh liver), adjusted to pH 1 with H_2SO_4, was extracted six times with 800 ml. portions of amyl alcohol. The combined extracts were then shaken three times with 1 l. portions of water containing enough NaOH to make the aqueous layer alkaline to thymol blue. The combined aqueous extracts were neutralized with HCl, evaporated *in vacuo* to about 100 ml. and treated with 4 vol. of 96 % alcohol. The precipitated salts were filtered, and the filtrate, after removal of alcohol *in vacuo*, was adjusted to 800 ml. (1 ml. = 6 g. of fresh liver).

Liver eluate fraction. To the residue from the amyl alcohol extraction of liver residue I diluted with 3 vol. of water (now 1 ml. = 2·5 g. fresh liver) and readjusted to pH 1·2, 150 g. of fuller's earth were added. After stirring for 30 min., the adsorbate was collected on a Büchner funnel and washed thoroughly with $N/10$ HCl; it was then twice eluted with 1 l. and 500 ml. portions of 2 % $Ba(OH)_2$. To the combined eluate, freed from Ba with H_2SO_4 and adjusted to pH 8 with NaOH, an excess of basic lead acetate (25 g.) was added. The resulting precipitate was filtered, and the lead removed from the filtrate with H_2S. The filtrate was then reduced in volume *in vacuo* to 200 ml. (1 ml. = 25 g. fresh liver).

Comparison by the rat growth method, of the eluate and filtrate fractions from liver with those from yeast

The rats used in these growth tests were prepared in the usual manner. After the usual depletion period of 1 week during which they received supplements of cod liver oil and aneurin, each male rat was given the additional daily supplements of 50 μg. of riboflavin and either yeast filtrate fraction purified by amyl alcohol extraction equivalent to 2 g. dry yeast or the above liver filtrate fraction equivalent to 6 g. fresh liver; each female rat received riboflavin and either yeast

eluate fraction equivalent to 2 g. dry yeast or the above liver eluate fraction equivalent to 12 g. fresh liver.

The two groups of male rats receiving the filtrate fractions increased in weight at approximately the same rate during the 2-week period during which they received this diet (see Table II). The average weight increases of the animals in

Table II. *Comparison of eluate and filtrate fractions from liver with those from yeast*

Each rat received daily 10–15 μg. aneurin and 50 μg. riboflavin. The supplements indicated in the table were given daily in the following equivalents: liver filtrate fraction = 6 g. fresh liver; liver eluate fraction = 12 g. fresh liver; yeast filtrate fraction, purified by amyl alcohol extraction = 2 g. dry yeast; yeast eluate fraction = 2 g. dry yeast.

No. of rats	Sex	Daily supplement given during preliminary period	Av. weekly wt. increase of group for preliminary period of 2 weeks g.	Additional daily supplement given during subsequent period of 2 weeks	Av. weekly wt. increase during subsequent period of 2 weeks g.
2	♂	Liver filtrate fraction	19, 13	Liver eluate fraction	32, 23
2	♂	Liver filtrate fraction	22, 20	Yeast eluate fraction	27, 26
5	♂	Yeast filtrate fraction	17, 14	Liver eluate fraction	25, 24
6	♂	Yeast filtrate fraction	18, 12	Yeast eluate fraction	24, 25
8	♀	Liver eluate fraction	16, 10	Liver filtrate fraction	25, 27
2	♀	Liver eluate fraction	22, 12	Yeast filtrate fraction	22, 21
10	♀	Yeast eluate fraction	20, 10	Liver filtrate fraction	24, 18
6	♀	Yeast eluate fraction	21, 13	Yeast filtrate fraction	22, 21

the two groups receiving the eluates from yeast and liver for the 2-week period were also the same. Certain of the rats of the two groups which had received the different filtrate fractions were now each given an additional daily supplement of yeast eluate fraction (= 2 g. dry yeast) and the others were given the liver eluate fraction (= 12 g. fresh liver). In all cases a marked increase in the growth rate resulted, and all animals continued to increase in weight at approximately the same rate during the 2-week test period, irrespective of the sources of filtrate and eluate fractions in their diets.

The rats which had received the eluate supplements during the first 2 weeks were given supplements of the filtrate fractions: certain animals of the two groups were each given doses of yeast filtrate fraction (= 2 g. dry yeast) and the others were given the liver filtrate fraction (= 6 g. fresh liver). Again, rises in the growth rates of all animals occurred, the weight increases being the same whether the animals received their fractions from yeast or liver.

The above experiments prove beyond reasonable doubt that liver filtrate and liver eluate fractions, prepared as described, contain the same dietary essentials for the rat as the fractions prepared from yeast.

Preparation of liver eluate and filtrate fractions from other liver residues

Liver eluate fraction from liver residue II. The liver residue II was treated with fuller's earth without previous extraction with amyl alcohol; the adsorbate was eluted with Ba(OH)$_2$ and the eluate purified by treatment with basic lead acetate as described above. When tested on rats this fraction proved to be contaminated with filtrate factor, which, however, was easily removed by extraction of the preparation with amyl alcohol. The product had then the same growth-promoting properties for rats as the yeast eluate fraction.

Liver filtrate fraction from liver residue III. Amyl alcohol extraction of liver residue III by the method described for the preparation of liver filtrate fraction from liver filtrate I, yielded a preparation with the same growth-promoting properties as the yeast filtrate fraction.

DISCUSSION

Considerable confusion in the literature concerning the vitamin B complex has resulted from the indiscriminate use of yeast, liver, rice bran, wheat germ and other materials as sources of factors in the complex. Fractions prepared in the same way from different materials have sometimes been finally proved to have quite different biological properties. Confusion has also been caused by certain authors assuming that effects noted in different experimental animals after deprivation of the same fraction are due to lack of the same factor. As each additional factor in the vitamin B complex is discovered, the need for caution in generalizing conclusions drawn from such results becomes more apparent.

In this laboratory we have realized for some time that the dietary essentials of the vitamin B complex contained in yeast fuller's earth filtrates are by no means always the same as those contained in liver fuller's earth filtrates. Experiments on pigs [Chick *et al.* 1938] and dogs [Koehn & Elvehjem, 1936] have proved that, while yeast fuller's earth filtrates contain little or no nicotinic acid or other pyridine derivative with similar biological properties, liver fuller's earth filtrates are rich in these substances. The marked difference, reported in this paper, between the fuller's earth filtrates as sources of rat dietary factors, cannot be due to the difference in the amounts of nicotinic acid derivatives they contain, since we have not been able to show that nicotinic acid or its derivatives have growth-promoting properties for rats [Macrae & Edgar, 1937]. Lepkovsky *et al.* [1936] reported that the preparation of liver fuller's earth filtrates free from factor I (yeast eluate factor) was comparatively easy, but it is possible that in our experiments all of that factor was not removed from the liver filtrate. Further biological investigation of liver fuller's earth filtrate fraction must be carried out before the relationship of the dietary factors contained in it to those of other vitamin fractions is clarified; the proof of the biological identity of one fraction from liver with our yeast filtrate fraction is one step in that direction. Since extractability from acid aqueous solution is a common property of the rat filtrate factor and the chick antidermatitis factor [Elvehjem & Koehn, 1935], the probability that these factors will eventually prove identical is increased. The relationship between our rat dietary factors and those required for growth and maintenance of pigeons [Carter & O'Brien, 1937] is an interesting problem which will probably be solved in the near future.

Only when all factors of the vitamin B complex are obtained in a pure state will the vitamin B problem be finally solved. The isolation of yeast eluate factor (vitamin B_6, factor I) in a pure state [Lepkovsky, 1938; Keresztesy & Stevens, 1938; György, 1938; Kuhn & Wendt, 1938; Edgar, El Sadr & Macrae, unpublished] has proved an important step. Since the crystalline vitamin completely replaces our eluate fraction in the diet of the rat, it seems probable that vitamin B_6 is the only essential nutrient for the rat present in our yeast and liver eluates. The possibility, however, still exists that other essential nutrients may be common contaminants of our eluate fraction, our filtrate fraction and even our basal diet.

SUMMARY

1. Fuller's earth treatment of liver extracts did not yield fractions with the same dietary essentials for the rat as did similar treatment of autoclaved yeast extracts.

2. Fractions with the same growth-promoting properties for rats as the yeast eluate fraction and yeast filtrate fraction were obtained from liver extracts by other methods.

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CCLXXXVII. THE ISOLATION OF A FLAVO-PROTEIN FROM COWS' MILK

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THE starting-point of this investigation was the observation that concentrated solutions of the xanthine oxidase of milk were characterized by a pronounced orange coloration. We have been able to isolate a flavoprotein compound which accounts for some though not all of this colour. There are, very likely, other flavoprotein compounds in milk besides the one to be described.

I. *Isolation*

(1) Fresh untreated cows' milk is warmed to 35° and treated with rennet as described by Dixon & Kodama [1926]. The curd is broken up and filtered through muslin. The filtrate is mixed with 1·1 vol. of sat. $(NH_4)_2SO_4$. The precipitate rises to form a sharp layer, and the fluid underneath is sucked off and discarded. The layer of precipitate is filtered with suction on Büchner funnels. The well-packed cake of precipitate is dried *in vacuo* over H_2SO_4 and the fat is extracted with ether. The yield is *ca.* 700 g. from 100 l. of milk. The powder keeps indefinitely when stored dry.

(2) A 10% solution of the powder is cooled to 0° and mixed with 0·56 vol. of sat. $(NH_4)_2SO_4$. Glacial acetic acid is added with vigorous stirring until the pH is 4·0 (just yellow to bromocresol green). The precipitate is centrifuged, and mixed with water equal in volume to the discarded supernatant fluid. After neutralizing with NaOH, the total volume is measured and the degree of saturation of the solution with respect to $(NH_4)_2SO_4$ is calculated on the assumption that the cake of precipitate is 36% saturated. 0·1 vol. of ethyl alcohol is added with vigorous stirring followed by sufficient sat. $(NH_4)_2SO_4$ to bring the degree of saturation to 25%. After acidification with glacial acetic to pH 4·0 the precipitate is centrifuged and discarded. The supernatant fluid is made 50% saturated with $(NH_4)_2SO_4$ and the precipitate filtered with suction and dried *in vacuo*. The yield is *ca.* 200 g. from 100 l. of milk.

(3) A 5% solution of the above powder is dialysed against running tap water for 12 hr. and mixed with 0·05 vol. of 25% basic lead acetate solution. The bulky precipitate is rapidly centrifuged and discarded. The relatively clear supernatant fluid is mixed with an equal volume of sat. $(NH_4)_2SO_4$. The centrifuged precipitate, which contains $PbSO_4$ and protein, is resuspended in 5 vol. of water. After neutralizing with NaOH, the suspension is centrifuged. The supernatant fluid which is retained has a pronounced orange colour. After dialysis against running tap water until free of $(NH_4)_2SO_4$, the solution is mixed with an equal volume of ethyleneglycol monoethyl ether and the pH adjusted to 3·8 by addition of glacial acetic acid. The precipitate is centrifuged and redissolved in the minimum quantity of $N/100$ NaOH. The solution is dialysed against running tap water and finally

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against distilled water until free of the reagent. The yield at this stage is about 2 g. from 100 l. of milk.

(4) At 38 % saturation of $(NH_4)_2SO_4$ and pH 7·0 only part of the flavoprotein is precipitated together with a large amount of colourless material. The precipitate is discarded. The supernatant fluid is made 50 % saturated with respect to $(NH_4)_2SO_4$ and centrifuged. The above procedure is repeated twice. The orange-red precipitate is dissolved in the minimum volume of $N/100$ NaOH and the solution dialysed against distilled water until salt-free. The yield is ca. 0·25 g. from 100 l. of milk.

(5) Milk flavoprotein is readily adsorbed on alumina $C\gamma$ at pH 6–7 and can be eluted from the adsorbing agent with a mixture of $(NH_4)_2HPO_4$ and NH_3 according to the method of Weygand & Stocker [1937]. This method of purification is only successful after extensive concentration of milk flavoprotein. The minimum quantity of alumina necessary to adsorb flavoprotein completely from a 0·5 % solution is used and this quantity must be determined experimentally for each preparation. Two elutions are sufficient for extraction of at least 90 % of the adsorbed flavoprotein. The combined eluates are dialysed against distilled water. After two adsorptions the yield is ca. 0·12 g. from 100 l. of milk.

Table I contains a summary of the scheme of purification and of the degree of concentration at the various stages. Assuming a 95 % loss in the purification

Table I. *Preparation of milk flavoprotein*

Milk	Flavoprotein present in
Rennin treatment, 35°	Filtrate
↓ 50 % sat. $(NH_4)_2SO_4$	Precipitate
↓ Dried and extracted with ether	Precipitate
↓ 38 % sat. $(NH_4)_2SO_4$ pH 4·0 and 0°	Precipitate
↓ 25 % sat. $(NH_4)_2SO_4$ pH 4·0 and in 10 % alcohol	Filtrate
↓ 50 % sat. $(NH_4)_2SO_4$	Precipitate
↓ Precipitate dried and dialysed (0·01 % flavinphosphate)	—
↓ 5 % solution treated with 0·05 vol. 25 % basic lead acetate	Filtrate
↓ 50 % sat. $(NH_4)_2SO_4$	Precipitate
↓ Precipitate redissolved and dialysed after centrifuging off $PbSO_4$	Filtrate
↓ Treated with equal volume ethyleneglycol monoethyl ether (0·1 % flavinphosphate)	Precipitate
↓ Redissolved precipitate dialysed and 38 % sat. $(NH_4)_2SO_4$ at pH 7·0	Filtrate
↓ 50 % sat. $(NH_4)_2SO_4$ (0·2 % flavinphosphate)	Precipitate
↓ Repeat twice the 38 and 50 % sat. $(NH_4)_2SO_4$ procedures (0·35 % flavinphosphate)	—
↓ After dialysis, adsorb on alumina $C\gamma$ and elute twice with $(NH_4)_2HPO_4$ and NH_3. Dialyse. Repeat process (0·53 % flavinphosphate)	—

there should be 2·4 g. of flavoprotein in 100 l. of milk. Since each litre contains 120 g. dry weight, the degree of concentration at the 0·53 % flavinphosphate stage should be 12,000/2·4 = 5000. It is difficult for various reasons to determine directly the initial concentration of flavoprotein in milk. Most important is the fact that fresh milk and the whey powder contain an enzyme which inactivates coenzyme I. Since the presence of flavoprotein at these stages can only be detected by a catalytic test, involving the use of coenzyme I, obviously no accurate estimation of concentration is possible. In addition to this complication, there is the fact that the concentration of flavoprotein in milk lies within the limits of error of the method of detection. The estimation of the loss in the purification process, particularly in the early stages, is approximate and merely gives order of magnitude. Stages 1 and 2 together involve about a 30 % loss mainly due to the bulkiness of the precipitate at 25 % saturation of $(NH_4)_2SO_4$ and pH 4·0. Stages 3, 4 and 5 involve losses of approximately 75, 60 and 30 % respectively.

A preparation of flavoprotein at the 0·53 % flavinphosphate stage is not homogeneous in an ultracentrifugal field. The amount of the impurity and the theoretical % of flavinphosphate which a homogeneous preparation should contain are discussed in the appendix by J. St L. Philpot. The low initial concentration of flavoprotein in milk and the great losses in the purification procedure render attempts at further purification extremely expensive. Starting with 50 l. of milk, one practically reaches the vanishing point at the 0·53 % flavinphosphate stage. In the final stages, scarcity of material is the limiting factor in the purification.

II. *Physical and chemical properties*

A concentrated solution of purified milk flavoprotein (> 10 mg./ml.) is orange-red in colour. Dilute solutions (< 2 mg./ml.) appear brownish yellow. On addition of hyposulphite to a neutral solution of the protein, the colour is bleached and restored by shaking with air. Re-oxidation is practically instantaneous as soon as all the hyposulphite is oxidized to sulphite.

Milk flavoprotein is rapidly denatured at room temperature in solutions of pH 3·8 or less, likewise in concentrated aqueous solutions (> 40 %) of methyl alcohol and acetone. Temperatures higher than 50° denature the protein within a few min. Denaturation is always accompanied by the liberation of the prosthetic flavin. Indeed the appearance of greenish fluorescence serves as a delicate indicator of denaturation. Loss of catalytic activity can be used as a quantitative measure of denaturation of flavoprotein. No success has yet been attained in the reversible resolution of flavoprotein into prosthetic flavin and undenatured protein.

The absorption spectrum of our purest flavoprotein preparation (0·53 % flavinphosphate) is given in Fig. 1. There are three main absorption bands with maxima at 279, 350 and 450 mμ respectively. The spectrum was recorded by a Hilger "Spekker" photometer. The positions of the band peaks are identical in all preparations examined although with purification the ratio

$$\log \frac{I_0}{I} (279 \text{ m}\mu) \Big/ \log \frac{I_0}{I} (450 \text{ m}\mu)$$

increased.

The spectrophotometric method was used to estimate the flavin content of preparations of known dry weight. The assumption was made that the absorption coefficient β at 450 mμ was $2·4 \times 10^7$. The justification for this assumption is that all known flavin-protein compounds have the same β value for their main

absorption band in the visible range of the spectrum. Since $\beta = \frac{1}{c} \cdot \frac{1}{d} \cdot \ln \frac{I_0}{I}$ then by determining the value of $\ln \frac{I_0}{I}$ at 450 mμ in a 2 cm. cell, the concentration of riboflavin, c, in mol. per ml. can be calculated from the formula. The concentration was always expressed in terms of g. % of flavinphosphate.
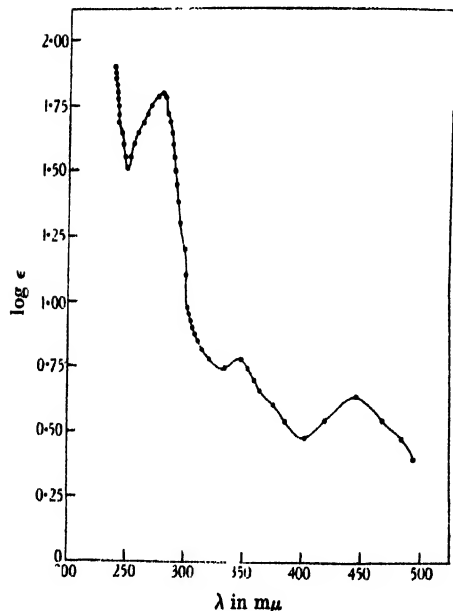
Fig. 1.
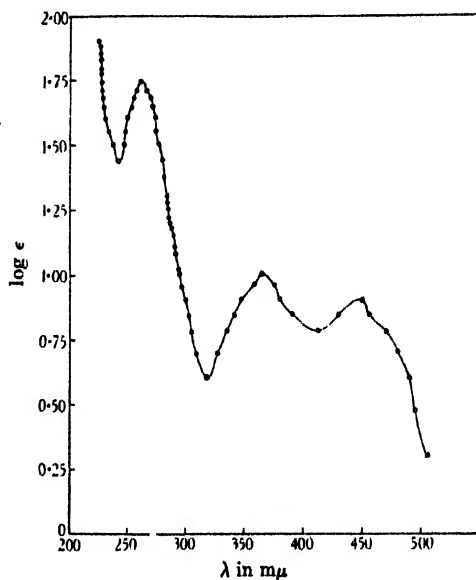
Fig. 2.

Fig. 1. Absorption spectrum of 1·76 % flavoprotein solution at the 0·53 % flavinphosphate stage; pH 7.

Fig. 2. Absorption spectrum of the prosthetic flavin after precipitation as the Ag and Ba salts; pH 7.

Estimation of the flavin content both directly in the combined form and indirectly after extraction with 75 % methyl alcohol from the protein shows that the yield of free flavin is about 80 %. We are satisfied that this small discrepancy is referable (1) to the inefficiency of extraction, and (2) to the marked tendency of the prosthetic flavin to become adsorbed on the protein precipitate. Fig. 2 shows the absorption spectrum of a purified sample of the prosthetic flavin. There are three main absorption bands with peaks at 260, 365 and 450 mμ. The spectrum is similar to, but not identical with, that of riboflavinphosphate.

III. *Prosthetic flavin*

By extracting a dry preparation of flavoprotein with 75 % methanol, the prosthetic flavin is obtained in solution and the protein is denatured. After removing methyl alchohol by distillation *in vacuo*, and precipitating residual denatured protein with $(NH_4)_2SO_4$, a clear yellow highly fluorescent aqueous solution is obtained. The prosthetic flavin is insoluble in benzyl alcohol; hence it cannot be riboflavin [cf. Emmerie, 1938] and is more probably a phosphorylated derivative. Cataphoretic measurements kindly carried out by Dr R. A. Kekwick of the Lister Institute show that the prosthetic flavin migrates anodically in a

cataphoretic field more rapidly than lactoflavinphosphate. At pH 7 in phosphate buffer, at $0°$ and $\mu = 0.02$ the mobilities of lactoflavinphosphate and the prosthetic group were, respectively, 1.8×10^{-5} and 2.4×10^{-5} cm.^{2}/volt^{-1}/sec.$^{-1}$. To quote Dr Kekwick: "these measurements are not very accurate owing to the large diffusion constants of the material, but it is safe to say that the unknown flavin migrated the faster". It was therefore clear that the prosthetic group was a phosphorylated derivative not identical with lactoflavinphosphate.

Straub [1938] and Warburg & Christian [1938] have demonstrated that the coenzyme of the amino-acid oxidase is a flavin-adenine dinucleotide. With the assistance of Dr Straub, we have been able to show that the prosthetic group of milk flavoprotein can act as the coenzyme of the amino-acid oxidase (cf. Table II).

Table II

The quantities used were: 0.7 ml. flavin-free amino-acid oxidase prepared by an unpublished method of Dr Straub; 1 ml. phosphate buffer, pH 7.2; 0.2 ml. M d-alanine. Total vol. 2.9 ml. Air in gas space.

Flavin added as μg. flavinphosphate per ml.	0	0.16	0.4	0.8	2
μl. O_2/10 min.	0	36	69	108	108

The high specificity of oxidation coenzymes in general argues the close chemical similarity of the prosthetic flavin and the coenzyme of the amino-acid oxidase, but biological interchangeability is by no means conclusive evidence of identity. In collaboration with A. H. Gordon and S. Williamson we are pursuing further the question of its relation to the amino-acid oxidase coenzyme. The physical and chemical properties of the two compounds do not seem to be identical.

IV. Catalytic properties

Reduced coenzyme I (diphosphopyridinenucleotide) is oxidized extremely slowly by methylene blue and other oxidation-reduction indicators. In presence of milk flavoprotein these reactions are enormously accelerated. The catalysis has been quantitatively measured in the following three test systems:

(1) reduced coenzyme + methylene blue;
(2) lactic dehydrogenase system + oxidized coenzyme + methylene blue;
(3) lactic dehydrogenase system + oxidized coenzyme + methylene blue (or pyocyanine) + O_2.

In systems (1) and (2) the velocity of the catalytic reaction is determined by measuring the rate of decoloration of methylene blue under anaerobic conditions. The difference between the two systems lies in the method of reducing the coenzyme. In (1) reduced coenzyme prepared by treating oxidized coenzyme with hyposulphite is used. In (2) the lactic dehydrogenase system is used to reduce oxidized coenzyme *in situ*. Since methylene blue is autoxidizable it is possible to study the reaction manometrically by measuring the rate of oxygen absorption as in (3). Apart from the different methods of measuring the reactions in (2) and (3) respectively, we are dealing in (2) with a steadily falling concentration of methylene blue and in (3) with a constant concentration of methylene blue. There should, therefore, be differences in the respective rates of reaction.

Table III shows the catalytic effect of flavoprotein on the reaction between reduced coenzyme I and methylene blue. The coenzyme was reduced by hyposulphite in $M/2$ NaHCO$_3$ solution according to the method of Green & Dewan [1937]. For the details of the Thunberg technique, cf. Green & Dixon [1934]. The

Table III

The quantities used were: 0·8 ml. of 1 % coenzyme solution and 0·2 ml. 0·0125 *M* methylene blue. Total vol. 2·5 ml.

Flavoprotein as μg. flavinphosphate	0	10	2	1	0·2
Reduction time in min.	15	0·5	0·9	1·5	2·1

rate of reduction of methylene blue by reduced coenzyme is within limits proportional to the concentration of flavoprotein. Increase of the concentration of flavoprotein beyond 4 μg./ml. of flavinphosphate has no influence on the catalytic rate under the conditions of the above experiment. It is also interesting that the efficiency of flavoprotein as a catalyst increases with dilution. In other words, the greatest efficiency is reached when the ratio

$$\frac{\text{concentration of reduced coenzyme}}{\text{concentration of flavoprotein}}$$

becomes very large.

In test system (2), the lactic dehydrogenase+lactate is used as the reducing agent of the coenzyme. The enzyme is prepared from rabbit skeletal muscle by the method of Green *et al.* [1937]. The product of oxidation, pyruvic acid, inhibits the reduction process and must be fixed with cyanide. Fig. 3 shows the dependence of the rate of methylene blue reduction on the concentration of flavoprotein. Above 4 μg. flavinphosphate per ml. increase of flavoprotein concentration effects no further increase in the rate of decoloration of methylene blue. As little as 0·04 μg. flavinphosphate per ml. is sufficient to demonstrate a positive catalytic test. No reduction occurs in absence of flavoprotein.
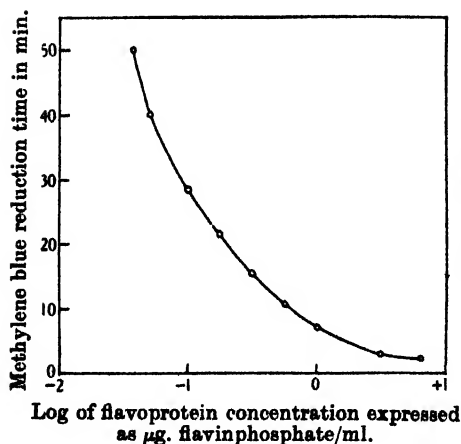
Log of flavoprotein concentration expressed as μg. flavinphosphate/ml.

Fig. 3.

Log of flavoprotein concentration expressed as μg. flavinphosphate/ml.

Fig. 4.

Fig. 3. The effect of the concentration of flavoprotein (expressed as μg. flavinphosphate) on the rate of reduction of methylene blue by reduced coenzyme.
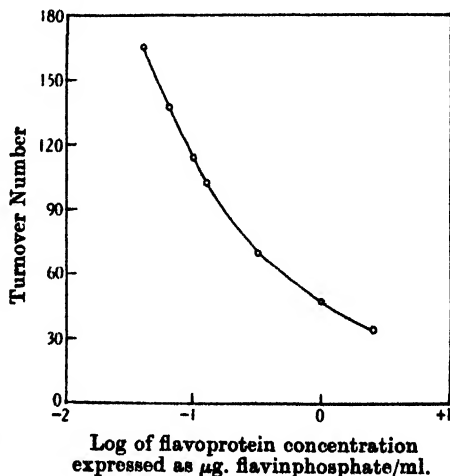
Fig. 4. The dependence of the Turnover Number on the concentration of flavoprotein (expressed as μg. flavinphosphate).

Assuming that flavoprotein undergoes a cycle of oxidation and reduction, thereby catalysing the reaction between reduced coenzyme I and methylene blue, one can easily calculate from the above data the number of times flavoprotein would have to be reduced or oxidized in 1 min. (Turnover Number) in

order to account for the overall reaction. The exact molarity of the methylene blue solution was 0·0125. Therefore 0·2 ml. of the methylene blue solution is equivalent to 56 μl. H_2. Also 1 μg. flavinphosphate is equivalent to 0·049 μl. H_2. The Turnover Number (T.N.) is equal to the hydrogen equivalent of the methylene blue reduced per min., divided by the hydrogen equivalent of the flavoprotein present. Fig. 4 shows the dependence of the T.N. on the concentration of flavoprotein. Under the conditions of the experiment a limiting value of about 165 per min. was reached.

In the above experiment, the concentration of coenzyme I was 0·38 mg./ml. The T.N. increases with the concentration of coenzyme. For example with 1 mg. coenzyme, 0·42 μg. flavinphosphate and 0·2 ml. 0·0125 M methylene blue in a total volume of 3·6 ml. the T.N. was 105. Repeating the experiment with 3·3 mg. coenzyme the T.N. was 294. Fig. 5 shows the dependence of the T.N. on the concentration of flavoprotein using 2·6 mg. coenzyme per ml. Under optimum conditions, i.e. high coenzyme and low flavoprotein concentrations, the limiting T.N. is about 550.

Concentration of flavoprotein in μg. flavinphosphate/ml.
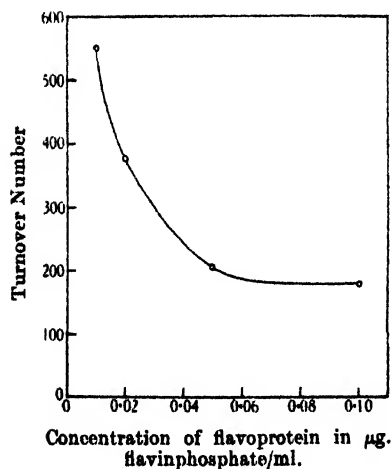
Fig. 5.

Fig. 6.

Fig. 5. The dependence of the Turnover Number on the concentration of flavoprotein using excess coenzyme. The quantities used were: 1 ml. enzyme; 1 ml. of 0·7 % coenzyme I; 0·2 ml. 2M HCN; 0·2 ml. 0·0125M methylene blue and 0·2 ml. M lactate.
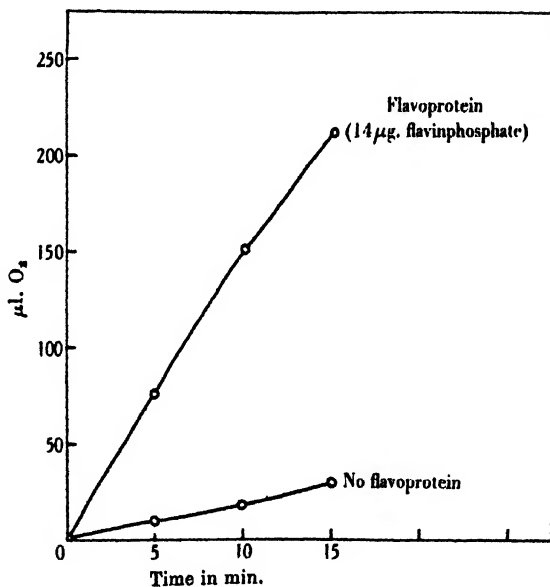
Fig. 6. The O_2 uptake of the lactic dehydrogenase system + pyocyanine + coenzyme I ± flavoprotein. The quantities used were: 1·5 ml. enzyme solution; 1 ml. 0·1 % coenzyme I; 0·2 ml. 2M HCN; 0·2 ml. 0·1 % pyocyanine and 0·1 ml. 2M lactate.

Table IV

The quantities used were: 1·5 ml. lactic dehydrogenase; 1 ml. 0·1 % coenzyme I; 0·2 ml. 2M HCN; 0·2 ml. 2M lactate; 0·2 ml. 0·1 % pyocyanine. Total vol. 4·1 ml.

ml. flavoprotein solution (14 μg. flavinphosphate per ml.)	1·0	0·3	0·1	0·03	0	1·0 (No pyocyanine)
μl. O_2/10 min.	152	94	43	31	22	28

Warburg & Christian [1932] have isolated a flavoprotein compound from yeast which also catalyses the oxidation of reduced coenzyme I by methylene blue. We compared the relative catalytic efficiencies of the two flavoproteins under identical experimental conditions. The ratio of activities milk flavoprotein : yeast flavoprotein was 7. This is in rough agreement with the limiting value of 50 for the T.N. of yeast flavoprotein, as compared with 500 for the milk compound.

The lactic dehydrogenase system + oxidized coenzyme + pyocyanine hardly absorbs oxygen. On addition of flavoprotein a vigorous uptake ensues (cf. Fig. 6). The dependence of the rate of O_2 uptake on the concentration of flavoprotein is shown in Table IV. The blank without flavoprotein is not inappreciable, a fact which rules out experiments with low concentrations of flavoprotein.

There is an unexpected result in Table IV, viz. that in absence of pyocyanine, flavoprotein shows no catalytic activity. In the previous section it was pointed out that reduced flavoprotein is autoxidizable. Two possibilities were open: either that reduced flavoprotein was not autoxidizable under the experimental conditions or that the assumption of flavoprotein undergoing a cycle of reduction and oxidation was incorrect. If the rate of oxidation of reduced flavoprotein by molecular O_2 was the limiting factor we should expect a higher rate of uptake in an atmosphere of O_2 than in air. Experiment showed that flavoprotein in absence of pyocyanine had no catalytic activity, regardless of whether an atmosphere of air or pure O_2 was used. The autoxidation of flavoprotein was therefore not the factor in question. If flavoprotein does not undergo a cycle of reduction and oxidation, it should be possible to demonstrate that flavoprotein is not reduced under the conditions of the experiment. The following experiments show that this is indeed the case. 8 mg. reduced coenzyme and flavoprotein in a concentration of $20\,\mu g.$ flavinphosphate per ml. were mixed anaerobically in a Thunberg tube at 38°. No decoloration of flavoprotein was observed in the course of 1 hr. In another tube, 0·2 ml. of 0·0125M methylene blue was added to the above mixture. Reduction of the methylene blue was complete in 30 sec. whereas the colour of oxidized flavoprotein persisted for more than 1 hr. Simple calculation shows that if flavoprotein were undergoing a cycle of reduction and oxidation, it would have to be reduced and oxidized 56/0·98 × 60) or 3400 times in 1 hr. in order to account for the observed rate of reduction of methylene blue. Yet even in 1 hr. there was no evidence of complete reduction. The same result was obtained when using the lactic dehydrogenase system + oxidized coenzyme instead of hyposulphite-reduced coenzyme. The colour of flavoprotein in these experiments was sufficiently intense to allow visual observation of changes in colour. A control tube with flavoprotein reduced by hyposulphite was used for comparison with the experimental tubes.

The above facts suggest the following picture of flavoprotein catalysis. Reduced coenzyme combines with flavoprotein. The pyridine ring in this complex is easily dehydrogenated by oxidation-reduction indicators such as methylene blue, pyocyanine etc. When oxidation of the pyridine ring takes place, the complex dissociates into oxidized coenzyme and the original flavoprotein. That is to say, flavoprotein remains in the oxidized state during the cycle of its catalysis. The cataphoretic method should easily decide whether such complex formation does in fact take place.

We have carried out similar experiments with the Warburg and Christian flavoprotein of yeast. Although this compound definitely undergoes a cycle of oxidation and reduction our calculations indicate that the cycle is not sufficiently rapid to account for the catalytic effect on the oxidation of reduced coenzyme I

by methylene blue. This may be due to the presence in our yeast preparations of the flavoprotein described by Haas [1938] which does not react directly with O_2.

It is possible to use many variations of the three test systems for demonstrating the catalytic action of milk flavoprotein. For example, the lactic dehydrogenase system can be replaced by other systems which require coenzyme I such as the malic, triose, triosephosphoric and α-glycerophosphoric systems. Similarly methylene blue or pyocyanine can be replaced by flavinphosphate or cytochrome c. There were indications that coenzyme I (diphosphopyridine-nucleotide) could be replaced by coenzyme II (triphosphopyridinenucleotide), but the experiments were not conclusive.

Preparations of flavoprotein at and beyond the 0·1% flavinphosphate stage of purity have no xanthine oxidase activity. There is no question, therefore, of the identity of flavoprotein with the xanthine oxidase.

V. *Catalysts for the oxidation of reduced phosphopyridinenucleotides*

There are four catalysts known to catalyse the oxidation of phosphopyridine-nucleotides. Table V summarizes essential information about their properties. Coenzyme factor is by far the most active catalyst of this group and is the only

Table V

	Reference	Source	Prosthetic group	Absorption bands mμ	Colour
Coenzyme factor	Dewan & Green [1938], Adler et al. [1937]	Animal tissues and yeast	Unknown	—	—
Warburg-Christian flavoprotein	Warburg & Christian [1932], Theorell [1934]	Yeast	Riboflavinphosphate	275, 380, 465	Yellow
Haas flavoprotein	Haas [1938]	Yeast	Dinucleotide of adenine and riboflavin	275, 377, 455	Greenish yellow
Milk flavoprotein	—	Milk	Unidentified flavin	279, 350, 450	Orange-red

one which has a wide distribution in animal tissues and micro-organisms. Nothing is known of its prosthetic group. All three flavoproteins differ in their physical and chemical properties. The differences are referable not only to the flavin but also to the protein portion.

SUMMARY

The isolation and properties of a flavoprotein and its prosthetic group from milk are described.

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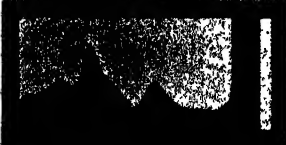
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ADDENDUM: EXAMINATION IN THE ULTRACENTRIFUGE

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Three specimens of milk flavoprotein at different stages of purification were sent by Messrs Corran and Green. The ultracentrifuge results are given in Table I. In every case the flavin appeared to belong wholly to a component having $S_{20} = 11-12 \times 10^{-13}$. Table I shows that in the purest preparation, containing

Table I

Photograph	Flavin-phosphate %	Sedimentation constants $\times 10^{13}$					Concentration of δ component % of total protein
		Refraction method				Absorption method	
		α	β	γ	δ	δ	
	0·06	1·1	3·9	6·6	11·5	*ca.* 11·0	—
	0·26	—	3·8	6·9	11·7	11·3	15–25
	0·53	—	—	6·2	12·1	12·5	39–46

0·53 % flavin (expressed as lactoflavinphosphate) the flavoprotein forms 39–46 % of the total protein. Hence the pure flavoprotein must contain 1·15–1·36 % flavin, and its mol. wt. divided by the number of flavin groups per mol. must be 34,000–40,000. In this it differs from yeast flavoprotein, which has one flavin group per mol. of weight 78,000 [Kekwick & Pedersen, 1936].

From the sedimentation constant 12×10^{-13} and, assuming the usual partial specific volume 0·75, the minimum mol. wt. (i.e. that of a spherical particle) is about 220,000. Hence there must be at least $220/40 = 5·4$ flavin groups per mol. Actually all known proteins with sedimentation constant about 12×10^{-13} have mol. wt. about 280,000 [cf. Svedberg, 1937], which is sixteen times the approximate "Svedberg unit" 17,600. The figure 34,000–40,000 given above suggests that milk flavoprotein may contain one flavin group to every two Svedberg units. If it were composed in all of sixteen Svedberg units it would therefore have eight flavin groups in a molecule of weight 270,000–320,000. Until the homogeneous protein is available this seems the most reasonable assumption.

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CCLXXXVIII. STUDIES ON THE KETOGENIC ACTIVITY OF THE ANTERIOR PITUITARY

I. THE RELATION OF KETONAEMIA TO KETONURIA IN THE RAT

II. A METHOD FOR THE ASSAY OF THE KETOGENIC ACTIVITY

III. THE NATURE OF THE KETOGENIC PRINCIPLE

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BURN & LING [1930] were the first to demonstrate that the injection of anterior pituitary extracts into normal fasting rats is followed by an increased urinary excretion of acetone bodies. This observation has been repeatedly confirmed in the rat and a similar increase in the urinary excretion of these substances has also been found in the dog [Rietti, 1934] and guinea pig [Best & Campbell, 1938]. As may be anticipated there also occurs in susceptible animals a simultaneous increase in the blood acetone bodies and this has been found in the rat, dog, man [Anselmino & Hoffmann, 1931] and rabbit [Magistris, 1932; Mirsky, 1936; Best & Campbell, 1936].

The nature of this effect of anterior pituitary extracts has given rise to some differences of opinion. Some investigators have been inclined to attribute it to a separate "ketogenic principle" or fat metabolism hormone [Anselmino & Hoffmann, 1931; 1936; Black *et al.* 1934]. Since the fatty acids represent the chief source of these substances it is not surprising to find that pituitary extracts also cause an increase in liver fat and a decrease in the quantity of depot fat [Anselmino *et al.* 1935; Best & Campbell, 1936; Fry, 1937]. However, as Best & Campbell [1936] have pointed out, wide discrepancies may exist in the magnitude of the ketonuria and the accumulation of liver fat under the influence of these extracts.

While the existence of a separate anterior pituitary principle specifically concerned with the mobilization and metabolism of fatty acids forms an attractive hypothesis for the interpretation of the experimental facts, there are other considerations of the action of anterior pituitary extracts that should be borne in mind before such a view is generally accepted.

The discovery [Houssay & Biasotti, 1931] that hyperglycaemia and glycosuria may be produced in fed dogs by crude anterior lobe extracts has been amply confirmed and since one explanation of their results is that such extracts suppress the utilization of carbohydrate [Russell, 1938], it would not be surprising if under such circumstances an increased proportion of fatty acids was utilized to meet the energy requirements. Furthermore, these crude extracts also produce marked effects on the protein metabolism of normal fed dogs.

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Teel & Cushing [1930] and Gaebler [1933] have shown that such injections produce not only a fall in the blood non-protein N but also a very striking decrease in the urinary N excretion. Consequently, it would seem that an equally strong case could be made for the view that the occurrence of ketonuria and an increased liver fat content is a secondary effect induced by an anterior pituitary hormone (or hormones) whose main influence is directed upon the carbohydrate and protein metabolism of the organism.

The above difficulty would be resolved if it could be shown that it is possible to isolate from the anterior pituitary gland fractions that independently alter the metabolism of fat, protein and carbohydrate. Anselmino & Hoffmann [1934] have claimed that by selective ultrafiltration at different H+ concentrations such a separation is possible. They report that one such protein-free fraction will increase the blood acetone bodies without affecting liver glycogen while another causes a decrease in liver glycogen without increasing blood acetone bodies. Magistris [1932] has also reported that the ketogenic principle of anterior pituitary extracts is ultrafilterable. Recently, in a series of preliminary communications, workers in Collip's laboratory have reported that not only is the ketogenic principle ultrafilterable but also that it is heat-stable at pH 10 and that they have obtained evidence that this activity is either identical or closely associated with the melanophore-expanding principle of the pituitary [Neufeld & Collip, 1938: O'Donovan & Collip, 1938; Denstedt et al. 1938]. Of even greater interest is their statement that this principle will also increase the glycosuria of hypophysectomized and depancreatized dogs, and raise the O_2 consumption and depress the R.Q. in both the normal and thyroidectomized rabbit. Finally, it may be pointed out in this connexion that although both Anselmino & Hoffmann and Magistris report that their ketogenic substance is ultrafilterable they find that the activity is destroyed by heating to 60° for 15 min.

The present paper deals with an attempt to establish more exactly the nature of this ketogenic action of anterior pituitary extracts. Unexpected difficulties were, however, encountered in the development of a reliable assay method for this activity based either upon alterations in the blood or urine ketone bodies, and since these observations have a definite bearing on the main problem they will also be considered at this time.

I. THE RELATION OF KETONAEMIA TO KETONURIA IN THE RAT

The success of any fractionation of the ketogenic activity of anterior pituitary extracts is dependent on the development of an accurate and reproducible method of assay.

The injection of crude saline or alkaline extracts into normal animals is followed by increases in the blood and urine acetone bodies that are mainly dependent on the sex, species and dietary condition of the animal used. Our own experience as well as that of others has indicated that the white rat of 120–150 g. initial weight does not excrete appreciable quantities of acetone bodies over a period of 72 hr. fasting, but the injection of anterior pituitary extracts produces in the majority of animals a well-marked ketonuria. Unfortunately we have observed a great variability in the magnitude of the ketonuria evoked in the responsive animals. Moreover, about 25% of the rats developed no ketonuria,

even though they were drawn from the same stock and were of the same sex as the responsive members.

Such a variability of response, which obviously excludes the induction of ketonuria as a quantitative method of assay, strongly suggests either that certain rats are immune to the extract or else that a definite level of ketonaemia must be reached before ketonuria can be detected. The latter alternative has proved to be the correct one and the following experiments would appear to establish at least in the rat a definite renal threshold for the acetone bodies.

Methods

Male rats weighing 120–150 g. were used exclusively. Determinations of the total acetone-body content of the urine were made by the method of Van Slyke [1917] and of the blood by the method of Van Slyke & Fitz [1917] with slight modifications which permitted the analyses to be made on 3 ml. blood.

The animals were divided into four groups.

(1) The first group of 59 rats had been maintained on a diet of dog chow (26 % protein, 7 % fat, 67 % carbohydrate) before use. They were fasted for 48 hr. and the urinary excretion of acetone bodies was determined during the second 24 hr. of fasting. Following this the animals were exsanguinated under Nembutal anaesthesia and the blood analyses made by a micro-method for the determination of blood acetone bodies which will be described in Part II. A fairly constant level of these substances is reached after some 30 hr. of fasting and this is maintained practically unchanged at least until the 55th hr. Consequently a blood analysis at the end of 48 hr. of fasting should give a fair representation of the level that had prevailed during the preceding 24 hr.

(2) 31 rats which comprised the second group were injected after 24 hr. fasting with 0·5 ml. anterior pituitary extract of proved ketogenic activity and the urinary excretion of acetone bodies during the next 24 hr. was determined. Following this a second injection of 0·5 ml. extract was given and the blood acetone bodies determined 4–5 hr. later.

(3) While exception might be taken to this last procedure on the grounds that the blood samples were not taken during the period of urine collection, we have previously found that the 24-hr. urinary excretion of acetone bodies in a given rat, after the succeeding daily injections of extract, is reasonably similar on the two successive days. However, in order to leave no doubt on this point, in another series of 12 rats we have taken blood during the period of urine collection. The relationship between the blood and urine acetone bodies of these rats was in no way different from those treated as in the first series.

(4) The final small group consisted of 6 rats that had been maintained on a low protein, high fat diet prior to fasting (protein 11 %, fat 40 %, carbohydrate 43 %). Deuel et al. [1937] have shown that a diet of this nature markedly increases the ketonuria of rats when they are fasted. In this series the urinary excretion between the 24th and 48th hr. of fasting was determined as well as the blood acetone bodies at the end of this period.

Results

Table I contains the average values and the range of both the urine and blood acetone bodies for these four groups of rats with an additional group of fully fed rats. This table clearly shows that in the control of fasting animals, although the blood acetone bodies varied over a wide range, yet in no animal did the ketonuria exceed 3·8 mg. in the 24 hr. In both series of animals injected with anterior pituitary extracts, although the average blood level was approximately double

that of the controls, the variation in the blood level within both the control and injected groups would make it impossible to decide by a single determination whether or not a given injection had exerted a stimulating action on the formation of acetone bodies. Consideration of the urinary acetone body excretion leads to similar difficulties. Although no control animal excreted more than 4 mg. acetone bodies, yet in one injected group 64 % and in the other 83 % of the rats failed to excrete more than this quantity when injected with 0·5 ml. anterior pituitary extract, a dose which is five times that which will produce ketonuria in some of the animals. It is true that as progressively greater amounts of extract are injected a larger proportion of animals will exhibit ketonuria, but it may be concluded that neither the urinary excretion of ketone bodies nor a *single* determination of blood acetone bodies is an adequate criterion for the presence or absence of ketogenic activity in a given extract unless prohibitively large numbers of animals are used.

Table I

| | Treatment | No. of rats | 24 hr. excretion of acetone bodies | | Blood acetone bodies | | % with urine acetone bodies greater than 4 mg./24 hr. |
			Mean	Range	Mean	Range	
1	Controls. Fasting 24–48 hr.	59	1·3 ±0·1	0·5– 3·8	12·1 ±1·1	0·8–32·3	0·0
2	Fasting. Injected 0·5 ml. APE	31	8·8 ±2·1	0·5–49·1	23·2 ±1·9	0·5–44·4	36·0
3	Fasting. Injected 0·5 ml. APE	12	7·6 ±4·2	0·3–44·2	23·8 ±4·2	6·0–53·0	17·0
4	Fasting. High fat, low protein diet	6	55·6	35·2–72·7	32·5	25·0–37·6	100·0
5	Fully fed. Dog Chow diet	11	—	—	1·4	0·7– 1·9	—

The reason for the variability in ketonuric response becomes apparent when the blood level of acetone bodies in all four groups of animals is correlated with the urinary excretion (Table II). It will be seen that the urinary acetone body content does not rise above 5 mg./24 hr. until the blood level has reached a value of c. 25–30 mg. 100 ml. Beyond this point the excretion rises sharply. The existence of this urinary threshold explains the phenomenon of a complete lack of response in a proportion of fasting rats which have been given anterior pituitary extract. These rats happen to have a low initial level of blood acetone bodies and although the extract produces ketonaemia, the kidney threshold is not exceeded and hence no ketonuria follows the injection.

Table II. *Relation between blood and urine acetone bodies of control and injected rats*

Blood acetone bodies mg. %	No. of animals	Urine acetone bodies mg./24 hr.
0– 4·9	16	1·0 ±0·1
5·0– 9·9	21	1·1 ±0·1
10·0–14·9	8	1·2 ±0·2
15·0–19·9	17	1·9 ±0·4
25·0–29·9	15	6·7 ±2·4
30·0–34·9	9	38·0 ±9·3
35·0	7	28·5 ±6·1

It has previously been shown that acetone itself is not a threshold substance [Widmark, 1920; Briggs & Shaffer, 1921] and consequently this threshold effect

is due either to the behaviour of β-hydroxybutyric acid or to acetoacetic acid or both. The existence of a renal threshold for these substances would be in accord with the view that these compounds are normally utilized by the organism.

Finally, it may be pointed out that the view that the fasting rat does not develop ketosis since it does not exhibit ketonuria would appear to be incorrect inasmuch as the blood level during fasting may exceed that in the fed animal by some twenty-fold (Table I).

II. A METHOD FOR THE ASSAY OF THE KETOGENIC ACTIVITY

The methods used to detect the ketogenic activity of anterior pituitary extracts have hitherto consisted either of determining the ketonuria following their injection into fasted animals, usually rats [Burn & Ling, 1930; 1933; Butts et al. 1934; Black et al. 1934; Best & Campbell, 1936; Gray, 1938] by the determination of blood ketone bodies in single samples of blood some hours after the injection [Anselmino & Hoffmann, 1931], or by following the blood ketone curve of injected rabbits [Magistris, 1932].

Our results (Part I) indicate that none of these methods will decide with certainty whether or not a given extract possesses activity. Thus, while a large dose of an active extract will unmistakably increase the ketonuria of a fair proportion of animals yet smaller doses of the same extract will fail to indicate the presence of the active principle. On the other hand, a single determination on the blood would be of value were it not for the extreme individual variation in the fasting ketonaemia, in consequence of which a large number of determinations must be made on both fasting and injected animals before statistically significant conclusions can be drawn (Table I).

The most desirable method, without question, would be one in which the acetone body content of the blood could be determined on the same animal both before and at a given time after injection. However, since the reliable blood acetone methods which exist at present require the use of fairly large blood samples for each determination, only animals of sufficient size to yield a copious quantity of blood can be successfully used. Magistris [1932] has employed rabbits in this manner for the assay of ketogenic activity, but it has been our experience with the strains of rabbits at our command that even large doses of an active extract often fail to produce any marked increase in ketonaemia even though the animals are first fasted for 48 hr. Dingemanse [1936] has likewise had no success in producing ketonaemia by the injection of anterior pituitary extract in this species.

Since the fasting young rat is perhaps the animal most sensitive to this action of anterior pituitary extract it appeared necessary to devise a micro-method by which serial determinations could be made of the acetone body content in the quantities of blood that might be withdrawn from the tail (c. 0·2 ml.). The reagent described by Scott-Wilson [1911] is sufficiently sensitive to be used for the nephelometric determination of the acetone body content in blood samples of this magnitude provided that the oxidation and distillation for the conversion of acetoacetic acid and β-hydroxybutyric acid into acetone can be performed with reasonable uniformity and accuracy. This reagent has already been used in this manner for the determination of acetone bodies in larger quantities of blood by Marriott [1913] and Rietti [1937].

The method of Abels [1937] is adaptable to 0·2 ml. blood but is only applicable to the determination of acetone and acetoacetic acid which comprise not more than 20–30 % of the total acetone bodies.

The following method for the assay of the ketogenic activity of anterior pituitary extracts was evolved and has proved the only procedure which, in our hands, has yielded consistently satisfactory results.

Procedure

Male rats weighing between 120 and 150 g. which had been maintained on a stock diet of Purina Dog Chow before being used, were fasted for 48 hr.; 24 hr. is not sufficient because the ketonaemia does not reach a plateau until the 30th hour after withdrawal of food. We have not found the fed rat to be sufficiently sensitive to pituitary extract to use it for the assay.

An initial sample of blood is obtained as will be described below. The extract under examination is then injected intraperitoneally, and a second sample of blood withdrawn 4–5 hr. later. Although the rise in blood acetone bodies after the injection of an active preparation does not reach a maximum for about 8 hr., it is sufficiently near its peak within 4 hr. for the purposes of assay. A response has been considered positive only when the blood acetone body content has increased 4 mg./100 ml. or more above the control level.

It has also been observed that rats which have an initial fasting level of less than c. 3 mg./100 ml. are often relatively unresponsive and therefore rats which had a fasting ketonaemia of 2·5 mg./100 ml. or less were not included in any series for assay.

Determination of total blood acetone bodies in 0·2 ml. blood

Blood was obtained by section of the tip of the tail after preliminary exposure of the rat to the heat of an electric bulb. The blood was gently milked from the tail on to a watch glass coated with K oxalate, 0·2 ml. blood taken up into an accurately calibrated pipette and transferred at once to a 15 ml. graduated centrifuge tube containing 10 ml. $N/70$ H_2SO_4. After stirring with a fine rod and allowing to stand for 10 min., 0·2 ml. 10 % Na tungstate solution was added and the mixture again well stirred and allowed to stand for an additional 2–3 min. It was then centrifuged and all the clear supernatant fluid poured into the distilling flask (A) of the apparatus shown in Fig. 1. This apparatus was made entirely of pyrex glass with the glass joint carefully ground in order to eliminate the use of grease, the presence of which is not desirable. The volume of the precipitate adherent to the centrifuge tube was noted (0·8–1·0 ml.) and subtracted from the total volume in order to obtain the true quantity of filtrate analysed.
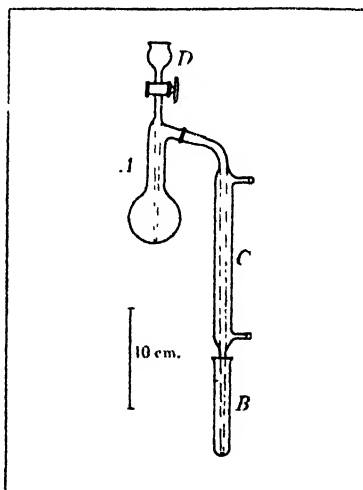
Fig. 1.

Two ml. 2 % $NaHSO_3$ were placed in the receiving tube (B) of the apparatus and the latter placed beneath the condenser (C) so that the outlet tube dipped almost to the bottom of the receiving tube.

The distilling flask was then heated with the flame of a shielded micro-burner. When the contents just reached the boiling point, the flame was slightly lowered

and 2 ml. 2 % K dichromate in 20 % H_2SO_4 to which a little talc powder had been added were run through the stopcock (D) which was then closed and the flame adjusted so that 5 ml. were distilled over into the receiving tube in the course of 20–30 min. It is advisable to calibrate the receiving tube so that distillation may be stopped when this volume has been collected. The contents of the receiving tube (7 ml.) were transferred to a 50 ml. Erlenmeyer flask and 5 ml. Scott-Wilson reagent added while the flask was whirled to insure uniform and rapid mixing. The receiving tube was then washed out with the mixture. Any delay or lack of thoroughness in the mixing will lead to erroneous and irregular results. Subsequent agitation should be avoided.

A series of standards for comparison with the unknown solution was prepared by placing 2 ml. $NaHSO_3$ in the flasks, then adding the requisite amount of a standard acetone solution and enough water to bring the volume to 7 ml. The Scott-Wilson reagent (5 ml.) was then added as above. A stock solution containing 1 mg. acetone per ml. may be kept in a closely stoppered bottle in the refrigerator for months without serious deterioration. One ml. of this solution was diluted to 50 ml. just before use; 1 ml. diluted solution is then equivalent to 10 mg./100 ml. of blood acetone. Standards equivalent to 5, 10 and 20 mg./ 100 ml. will suffice.

The unknown and the standard solutions are kept for 15 min. and then compared either in a colorimeter, using a blue filter, or else in a nephelometer.

The time of standing before comparison is of some importance since the turbidity develops rather slowly when quantities of less than 5 mg./100 ml. acetone are present. It reaches a maximum after approximately 20 min. with these low concentrations while, on the other hand, with concentrations above 5 mg./100 ml. the maximum turbidity is reached within 3–4 min. In the determination at high concentrations a period of standing beyond 5 min. causes some aggregation and consequently a slight diminution in turbidity; however, this error is not of serious magnitude below values of 25 mg./100 ml.

The method is sensitive to blood acetone body concentrations as low as 1·5–2·0 mg./100 ml. and, if desired, the preformed acetone and acetoacetic acid may be determined separately. To determine these 5 ml. 0·25 % H_2SO_4 are added to the blood filtrate in the distilling flask and a preliminary distillation carried out before the addition of potassium dichromate.

Results

In Table III are recorded a series of consecutive determinations of acetone and β-hydroxybutyric acid. Of the former 20 μg. were added to 12 ml water; of the latter, sufficient was added to whole blood to give initial concentrations of 5, 10 and 20 mg./100 ml. in terms of acetone.

Table III. *Recoveries of acetone and of β-hydroxybutyric acid*

20 μg. acetone in 12 ml. water. % recovery: 85, 87, 81, 92, 82, 90, 90, 84, 82, 85. Average 85 %

β-Hydroxybutyric acid expressed as acetone in blood sample mg./100 ml.	Converted and recovered as acetone %	Average %
5	77, 63, 73, 76	79
10	73, 65, 70, 75	71
20	72, 64, 64, 65	66

The average recovery of acetone is 85 %; while the conversion of β-hydroxybutyric acid into acetone averages 65–70 %, a value that compares favourably

with that obtained by other methods. The variability in the results is, in actual practice, of little consequence since the significant blood changes that are measured are always of a much greater order of magnitude. A combination of aeration and distillation will raise the recovery of acetone alone to 95 %, but leads to a still greater variability in the quantity of β-hydroxybutyric acid converted into acetone even when the rate of air flow is carefully controlled.

It was discovered that simple boiling of a very dilute solution of acetone ($20 \mu g.$ in 10 ml.) for 15 min. in a sealed glass container resulted in a 15 % loss. The low recovery of acetone by distillation is thereby explained. Aeration undoubtedly allows a higher recovery by virtue of the fact that the acetone is rapidly "washed out" at a temperature somewhat below 100°. In the determination of total acetone bodies a reduction in temperature during aeration would undesirably influence the oxidation of β-hydroxybutyric acid and thereby reduce the yield of acetone.

The use of this method to detect the ketogenic activity of small quantities of anterior pituitary extract is illustrated in Fig. 2. The activity of such quantities of extract would be difficult, if not impossible, to detect if reliance had to be
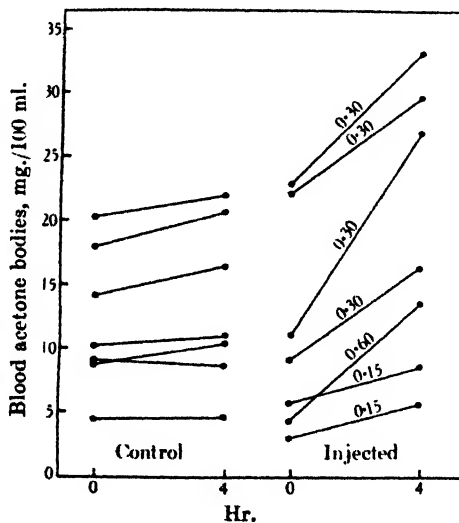
Fig. 2. The effect of anterior pituitary extract on the blood acetone bodies of fasted rats. The quantity in mg. of organic solids injected is indicated on the curves.

placed on single blood estimations or on the ketonuria over a period of 24 hr. A most gratifying aspect of the use of the method has been the relative constancy with which various animals show either a positive or negative response to small doses of a given extract.

III. THE NATURE OF THE KETOGENIC PRINCIPLE

The development of a sensitive method for detecting the ketogenic activity of anterior pituitary extracts (Part II) has enabled us to investigate certain claims that have been advanced as to the chemical nature of the substance responsible for this activity.

(a) *Heat-stability*. All workers with the exception of Neufeld & Collip [1938] have found the ketogenic principle to be heat-labile. Our results show that heating previously active extracts in the boiling water bath at pH 10 for 10 min. destroys their activity (Table IV).

Table IV. *Effect of heating at pH 10 in boiling water bath for 10 min. on the ketogenic activity of anterior pituitary extracts*

| | Amount injected | Blood acetone bodies (mg./100 ml.) | |
Extract	mg. protein	0 hr.	4 hr.
A, unheated	1·4	7·2	14·2
,,	1·4	6·6	11·0
heated	1·4	7·2	8·1
,,	1·4	2·8	3·0
B, unheated	0·3	9·8	16·9
,,	0·3	11·0	15·5
heated	2·0	10·4	10·1
,,	2·0	3·6	5·0

(b) *Ultrafiltration*. Anselmino & Hoffmann [1931; 1936], Magistris [1932], and more recently Denstedt *et al.* [1938], have reported that the ketogenic principle will pass through membranes that retain proteins, i.e. cellophane and 8 % acetic-acid-collodion. In our experience, however, this principle will not pass through such membranes. Our starting materials have been both aqueous extracts of acetone-dried glands and alkaline extracts of fresh glands. The filtration was carried out under suction at pH 8·0–9·0, and both the original ultrafiltrate and that concentrated fourfold *in vacuo* failed to influence the blood acetone body content of the fasted rat (Table V). However, the residue remaining in the sacs still possessed marked activity in much smaller doses than those given of the ultrafiltrate. The latter, although free from protein, still contained considerable quantities of N (0·2–0·25 mg. per ml.).

It is well known that membranes may be made with any desired pore size; those that we have used would neither permit proteins nor the substance responsible for the ketogenic activity of the anterior pituitary to pass. It would be necessary to employ a series of graded membranes in order to determine the

Table V. *Effects of (A) original extract, (B) its ultrafiltrate, and (C) a boiled extract of pituitary "colloid" on the blood acetone bodies of fasted rats*

| | Amount injected | | Melanophore expanding activity | Blood acetone bodies mg./100 ml. | | |
Extract	ml.	mg. N	frog units/ml.	0 hr.	4 hr.	% change
(A) Original	0·03	0·04	1,300	22·0	29·4	+ 34
,,	0·03	0·04	1,300	22·8	32·9	+ 44
,,	0·03	0·04	1,300	11·0	26·7	+143
,,	0·03	0·04	1,300	9·0	16·2	+ 80
(B) Ultrafiltrate	1·5	0·35	20	13·5	13·4	± 0
	1·5	0·35	20	8·4	7·8	− 8
Concentrated ultra-filtrate	1·5	1·4	200	22·9	24·1	+ 5
	1·5	1·4	200	8·8	7·0	− 20
(C) Pituitary "colloid"	1·0	—	10,000	14·9	17·0	+ 14
,,	1·0	—	10,000	4·0	2·5	− 38
,,	2·0	—	10,000	7·6	6·9	− 9
,,	2·0	—	10,000	3·9	3·7	− 5
,,	2·0	—	10,000	10·6	9·7	− 9
,,	2·0	—	10,000	9·3	11·0	+ 16

molecular size of this substance. Our findings suggest, however, that the molecule is a relatively large one.

(c) *The melanophore-expanding principle.* In view of the suggestion by O'Donovan & Collip [1938] and Denstedt *et al.* [1938] that the ketogenic activity is either identical or closely associated with the melanophore-expanding principle, we have prepared from 10 g. beef pituitary "colloid", by extraction with boiling 0·25 % acetic acid, an extract that contained 10,000 frog units per ml. of this principle. This extract, in doses of 1–2 ml., did not increase the blood acetone bodies of fasted rats (Table V) nor did it increase the glycosuria of partially depancreatized rats when administered in doses as large as 20,000 units, although these same rats responded to doses of crude anterior pituitary extract containing only 1000 units of melanophore principle.

The heat-lability of the ketogenic principle (Table IV) also speaks against its identity with the melanophore principle since the activity of the latter was somewhat increased by heating at pH 10 for 15 min. It may be mentioned that crude anterior pituitary extracts also lost their glycosuric activity in the partially depancreatized rat after heating, a confirmation of the report of Houssay & Biassotti [1931].

(d) *Fractionation of the protein of the anterior pituitary.* Inasmuch as the present evidence would indicate that the hormones of the anterior pituitary are either proteins or else closely associated with proteins, we have made various attempts to concentrate the ketogenic activity by simple protein fractionations. Neither isoelectric precipitation of alkaline or saline extracts, nor fractional precipitation by different concentrations of ammonium sulphate gave any clear-cut separation of active from inert material, since the ketogenic activity appeared to spread itself over a number of different fractions.

The following plan of fractionation was the most satisfactory in the sense that a fair degree of separation of the various pituitary principles was achieved and that a ketogenic fraction was obtained relatively free from certain of the other hormones (Table VI).

Table VI

CRUDE EXTRACT at pH 3·5
Acetone added to 80 % concentration

PRECIPITATE
(80 % acetone-insoluble)

FILTRATE
(80 % acetone-soluble)
Acetone concentration raised to 90 %

PRECIPITATE
Isoelectric separation

FILTRATE
(Discarded)

pH 6·8 INSOLUBLE
FRACTION

pH 5·5 INSOLUBLE
FRACTION
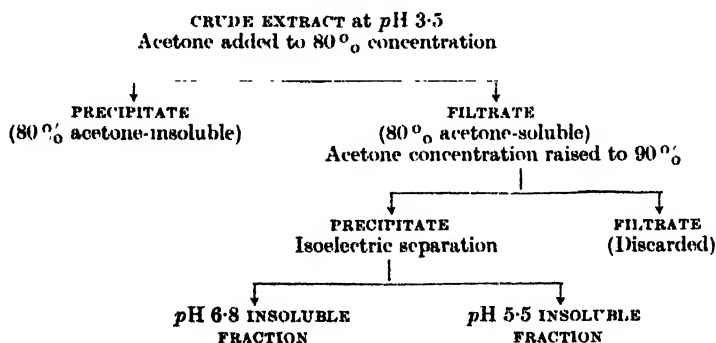
Four vol. acetone are added to a Burn & Ling extract [Burn & Ling, 1933] after bringing the pH to 3·5 with dil. HCl. If flocculation does not occur, a small amount of sat. NaCl is stirred in until there is a distinct separation of flocculent precipitate from clear solution. After centrifuging and filtering through hardened filter paper, the clear light brown filtrate is treated with an equal vol. acetone and allowed to stand about ½ hr. The precipitate is centrifuged off, dissolved in a vol. of water equal to ¼ of the original extract, and the pH brought to 6·8. The

isoelectric precipitate is taken up in water containing enough NaOH to bring to pH 10–11. Dil. HCl is then added until the pH reaches 7·5–8·0. There should be a distinct turbidity at this point, but no flocculation. The solution is then frozen, thawed and the greenish-gray precipitate centrifuged off; dissolved in alkali again and refrozen at pH 7·5–8·0, as before. For the final solution this precipitate is dissolved in alkali at pH 9·0, or acid at pH 5·5. The three supernatants from the above precipitations are pooled, the pH lowered to 5·5; and the precipitate, which may be enhanced in yield by freezing, dissolved in water and alkali and brought to pH 7·0. The final solutions may be preserved indefinitely if kept frozen at $-10°$ to $-20°$.

The acetone-insoluble fraction and the two acetone-soluble fractions were assayed for the familiar anterior lobe hormones in order to determine how the ketogenic potency was affected by the fractionation as compared with the other hormonal activities. The results are summarized in Table VII.

The methods which were used for assay were as follows: 100 g. hypophysectomized rats, 14–20 days after operation, were used for growth assay. The intracutaneous crop test of Lyons & Page [1935] was employed for assaying the lactogenic principle. The chick method of Smelser [1937] has proved both simple and reliable for the assay of thyrotropic activity. 21-day old male rats were used for adrenotropic assays [Moon, 1937] and gonadotropic activity was determined by the mouse uterus method as used by Levin & Tyndale [1937]. The familiar darkening of the bleached skin of the frog was utilized for the detection of melanophore activity. Ketogenic assays were performed as already described. The partially depancreatized rat has proved to be an excellent test object for the "diabetogenic" principle. Many of these rats do not exhibit a spontaneous glycosuria, but will readily respond after the injection of anterior pituitary extract. Although quantitative assays of the "diabetogenic" principle were not attempted, these animals serve as very satisfactory test objects to establish the presence or absence of the substance which is capable of producing hyperglycaemia and glycosuria. For the sake of convenience the various potencies are expressed

Table VII. *Relative hormonal potencies*

(Units per mg of organic solids.)

	Thyrotropic	Ketogenic	Diabetogenic	Growth	Lactogenic	Adrenotropic	Gonadotropic	Melanophore
Original Burn & Ling extract	10–15	4–8	Active	15–25	100	10 mg. produced 25 % increase in wt. of adrenals	--	1300
80 % acetone-insoluble fraction	30	—	—	—	—	—	--	—
80 % acetone-soluble pH 5·5 fraction	None*	4–8	Active	15 25	1000	- -	- -	—
80 % acetone-soluble pH 6·8 fraction	None*	4–8	Active	15–25	10	10 mg. produced 21 % increase in wt. of adrenals	None†	5

·* No response with 3·5 mg. † No response with 8·5 mg.

in terms of arbitrary units wherever possible. A unit refers simply to the minimal dose in terms of organic solids which produces an unquestionable response in the majority of test animals. With the thyrotropic, growth and adrenotropic assays units are expressed in terms of daily dose, otherwise the total dose is referred to.

The outcome of the series of assays was significant (Table VII) not only because of the fact that ketogenic and "diabetogenic" activities were seen to run parallel with each other, but also because their occurrence coincided with that of the growth principle. It had appeared from preliminary assays that the pH 5·5 fraction would be low in growth-promoting activity, but in the end it became quite clear that this fraction was fully as potent as any other.

The pH 6·8 isoelectric fraction, in addition to being free from thyrotropic principle, was also quite low in lactogenic principle, and contained no detectable gonadotropic activity. A moderate amount of adrenotropic activity was present and the very low content of melanophore principle in this fraction again speaks against this hormone being responsible for the ketogenic and "diabetogenic" effects of anterior pituitary extracts.

The assays are disappointing in the fact that they reveal that there has been no concentration of ketogenic activity as the result of the process of fractionation. While this may be due to a destruction of some of the active material during the fractionation procedure, the retention of ketogenic, growth and "diabetogenic" activity in equal amounts is a fact of some significance. Furthermore, the absence of all or most of the thyrotropic, gonadotropic, lactogenic and melanophore principles from the pH 6·8 fraction allows us to conclude that these hormones are not of chief importance in the production of these effects on metabolism.

Additional information as to the identity of the ketogenic principle is obtained by the assay of the two proteins obtained by the Lyons [1937] acid acetone extraction method for the preparation of prolactin (Table VIII).

Table VIII. *Relative hormonal potencies—Lyons acid acetone fractions*

	Lactogenic	Adrenotropic	Ketogenic	Glycosuric	Growth
pH 5·5 fraction	10,000 units per mg.	—	Less than ¼ unit per mg.	No response with 20 mg.	Less than ¼ unit per mg.
pH 6·5 fraction	—	10 mg. produced 29 % increase in wt. of adrenals	No response with 7 mg.	No response with 17 mg.	No response with 4 mg. per day

The pH 5·5 fraction has an exceedingly high content of lactogenic activity, while the pH 6·5 fraction, although not especially rich in adrenotropic activity, at least contained more of this principle than has been found in other preparations. Both these fractions, however, contained, at the most, only traces of growth, ketogenic and "diabetogenic" activities as judged by our methods of assay.

DISCUSSION

The practice of assuming that the multiple effects of anterior pituitary extracts upon metabolism are due to separate hormones does not seem desirable in the absence of any chemical evidence that this is the case. On the contrary, a consideration of the known facts of metabolism indicates that some of the alterations produced by the injection of anterior pituitary extracts may be regarded as secondary consequences of their action. Among these we suggest the production of ketosis may be included.

Our reasons for this assumption are not only our failure to confirm the claim of Anselmino and Hoffman that ultrafiltration will yield a product that specifically increases the acetone body content of the blood and urine, but are based on the contention that the well-substantiated action of anterior pituitary extracts on the carbohydrate and protein metabolism must of necessity increase the proportion of fat undergoing catabolism and, in consequence, increase the

formation of acetone bodies. Thus, it has been shown that the injection of these extracts into fed normal or hypophysectomized rats results in a decreased utilization of carbohydrate as evidenced by a fall in R.Q., an increase in blood sugar and in muscle and liver glycogen [Fisher *et al.* 1936; Meyer *et al.* 1937; Russell, 1938]. There is little reason to suspect that a similar action of anterior pituitary extracts in suppressing carbohydrate utilization does not occur in fasted rats, even though the utilization of carbohydrate under these conditions is reduced to a low level. Now, since it is in the fasted rat that the ketogenic action of this extract is most readily demonstrated, it may be suggested that this is a necessary consequence of its effect on carbohydrate utilization and is not due to a specific stimulation of fatty acid catabolism. Although it might be argued that a further reduction in the already low level of carbohydrate metabolism would not account for the magnitude of the observed ketosis it is necessary to point out that anterior pituitary extracts also decrease the proportion of protein undergoing catabolism. This is shown by the fall in blood non-protein N and urinary N excretion that follows their injection into fed animals [Teel & Cushing, 1930; Gaebler, 1933]. In addition, Lee & Schaffer [1934] have found that under these circumstances there is an increased retention of protein in the body. This effect has been attributed to the growth-promoting principle of the pituitary and is not associated with either the thyrotropic or gonadotropic principles [Gaebler, 1935, 1; 1938]. Furthermore, in the fed dog, Gaebler [1935, 2] has conclusively shown that this protein-sparing effect is accompanied by an increased utilization of fat. In the fasting rat, Harrison & Long [1938] have observed a 30–40 % reduction in urine N on treatment with anterior pituitary extracts rich in growth-promoting, "diabetogenic" and ketogenic activities. Consequently, although in the fasted rat a further reduction in carbohydrate utilization may be of little consequence, the removal of such a large proportion of protein from catabolism must increase the proportion of fatty acid catabolism and offers a satisfactory explanation for the ketosis and fatty infiltration of the liver that follows these injections.

This view of the mode of action of the anterior pituitary removes the necessity for postulating a hormone specifically concerned with fat metabolism and most certainly for a separate principle whose function is to increase the acetone body content of the blood and urine. Such a view is also in accord with our present inability to dissociate by chemical means the ketogenic activity of the anterior pituitary from its growth-promoting and "diabetogenic" activities. Even if the latter two effects are ultimately shown to be due to separate hormones it does not lessen the present argument that a decreased utilization of carbohydrate or protein must be reflected by an increased catabolism of fatty acids since the energy requirements of the organism must still be met.

Since, in the fasting animal, the sole source of carbohydrates is the tissue protein, this effect of the anterior pituitary in decreasing protein catabolism may be regarded as an advantage to the organism. In a larger sense there must exist some critical mechanism which determines whether a cell will store protein or catabolize that already present. In such a mechanism a hormonal factor, derived from the anterior pituitary, might be of primary importance.

SUMMARY

1. The existence of a urinary threshold for acetone bodies in the rat renders the development of ketonuria in response to anterior pituitary extracts in this animal unsuitable for purposes of assay. The increase in blood acetone-bodies will not give rise to ketonuria unless this urinary threshold is exceeded.

2. While the fed rat shows a negligible increase in blood acetone bodies in response to the injection of anterior pituitary preparations, the fasting rat responds quite readily. Fasting alone however produces a distinct but exceedingly variable ketonaemia. In consequence, single blood analyses made on injected rats are not reliable for the determination of ketogenic activity unless extremely large groups of animals are used.

3. A micro blood acetone body method is described which employs only 0·2 ml. tail blood, and thus allows a control blood acetone reading to be made on a given animal before injection of an extract. This method makes it possible to detect the presence of a small quantity of ketogenic principle and to determine the minimal effective dose.

4. The ketogenic principle could not be filtered through 8 % acetic collodion or cellophane. It was destroyed by heating on the boiling water bath for 15 min. at pH 10.

5. Comparative assays of relative potencies of the various anterior pituitary hormones present in a number of different preparations obtained by chemical fractionation, indicated that ketogenic activity followed growth and "diabetogenic" activity. This finding suggests that a single principle may be responsible for all of these three effects of the anterior pituitary on metabolism.

6. It is pointed out that the inhibitory action of anterior pituitary extracts on the catabolism of carbohydrate and protein offers a satisfactory explanation for the occurrence of the ketosis that follows their injection.

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CCLXXXIX. OBSERVATIONS ON
TISSUE GLYCOLYSIS[1]

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THE substances whose action will be described are mainly: (1) the ammonium ion, (2) glutamic acid and (3) maleic acid. They affect tissue glycolysis in different ways: either, as with (1), by raising the aerobic glycolysis and at the same time depressing anaerobic glycolysis, or, as with (2), mainly by inhibiting anaerobic glycolysis, or, as with (3), by a slow and continuous rise of the aerobic glycolysis to the normal level of the anaerobic glycolysis. The observed effects only occur or are specially pronounced in brain tissue.

Methods

The experiments were carried out at 37·5° in the Warburg apparatus. Tissue slices, suspended in bicarbonate or phosphate Krebs-Ringer solution containing 0·2 % glucose (pH 7·4), were used. In most experiments respiration and glycolysis were simultaneously measured by Warburg's differential method [1930]. Additions to the suspension medium were present from the beginning in most cases, so that the first reading was taken 15–20 min. after the tissue came in contact with the solution. For brain, embryo and medulla of kidney R.Q. of 1, for testis, intestinal mucosa and spleen R.Q. = 0·9 and for Jensen sarcoma R.Q. = 0·8 were assumed [cf. Dickens & Šimer, 1930; 1931; Dickens & Weil-Malherbe, 1936].

Estimations of lactic acid were made according to Friedemann & Kendall [1929] after copper-lime treatment, estimations of NH_3 by steam distillation *in vacuo* and subsequent Nesslerization according to Parnas & Heller [1924].

I. THE EFFECT OF AMMONIUM IONS

Ashford & Dixon [1935] found that addition of $M/10$ KCl to the Ringer solution increases both respiration and aerobic glycolysis of rabbit brain slices, while inhibiting their anaerobic glycolysis. Later, Dixon & Holmes [1935] and Dickens & Greville [1935] showed that the same effect can be obtained with CsCl and RbCl. The effect is absent from tumour, kidney, testis and yolk sac of the rat [Dickens & Greville, 1935] and from chick embryo [Needham *et al.* 1937]. According to Dixon [1937] the effect on brain tissue is seen equally well with $M/20$ KCl, though it is only small at $M/100$.

NH_4Cl affects respiration and glycolysis of brain tissue in a very similar way: $M/30\ NH_4Cl$ causes a rise of respiration as well as of aerobic glycolysis up to the

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normal level of the anaerobic glycolysis, while at the same time inhibiting the anaerobic glycolysis. As with KCl this effect is, with few exceptions, restricted to brain tissue (Table II). In spleen an increased aerobic glycolysis was found. An effect comparable with that in brain was also observed with intestinal mucous membrane in one experiment. But in another experiment with the same tissue this effect was very small and in a third experiment inhibition of both respiration and glycolysis occurred. The figures for the metabolism of the control were very similar to each other in these and other experiments.[1] With several other glyco-lysing tissues inhibition of anaerobic glycolysis by $M/30$ NH$_4$Cl was observed, but the effect on respiration and aerobic glycolysis was negligible or, at most, a slight suggestion of that found with brain.

The action of NH$_4$+ on brain however differs in one respect from those of K$^+$, Cs$^+$ or Rb$^+$. Whereas in these cases concentrations $> M/100$ are apparently necessary to produce an effect, the effect of NH$_4$+ is still recognizable at concentrations as small as $0.3 \times 10^{-3} M$ (Table 1), which still cause a marked increase of aerobic glycolysis and also a small stimulation of respiration. Low concentrations ($M/1000$ and less) sometimes seem to stimulate the anaerobic glycolysis as well; but in other experiments, reported in Table XI, inhibition was still observable at $M/1000$.

Table I. *The effect of NH$_4$+ on brain metabolism. Slices of guinea-pig brain (grey matter) in bicarbonate-saline*

	Concentration $10^{-3} M$ NH$_4$Cl	Q_{O_2}		$Q_G^{O_2}$		$Q_G^{N_2}$
		1st hr.	2nd hr.	1st hr.	2nd hr.	1st hr.
(1)	33.3	− 19.5	− 10.2	19.2	12.1	4.4
	10	− 11.6	− 9.0	8.0	6.1	6.0
	3.3	− 11.8	− 8.6	11.2	8.8	19.6
	1	− 10.6	− 8.7	9.2	7.2	26.0
	0	− 6.9	− 5.8	3.1	0.6	20.4
(2)	1	− 10.8	− 8.8	10.2	7.6	16.0
	0.33	− 9.3	− 6.3	6.8	2.5	15.9
	0.1	− 7.0	− 6.3	4.0	1.0	17.8
	0	− 6.8	− 6.0	4.4	0.9	16.8

Dickens & Greville [1935] explained the K$^+$ effect as the result of a disturbance of the ionic balance of the medium leading to physical changes in the colloidal structure of the cell protoplasm and probably to increased permeability. The same may be true for NH$_4$+ in high concentration, but with NH$_4$+-concentrations of the order of $10^{-3} M$ a specific toxic effect seems more likely.

II. The effect of glutamic acid

(a) Action on the anaerobic glycolysis

During his studies on glutamine formation in nervous tissues Krebs [1935] discovered the inhibition of anaerobic lactic acid formation by glutamic acid. On his suggestion I investigated the phenomenon in greater detail.

$l(+)$Glutamic acid, in a concentration of $M/100$, inhibits the anaerobic glycolysis of brain slices by 30–70 %. The inhibition increases with time and

[1] The figures reported here for the normal metabolism of rat intestinal mucous membrane differ from those found in the literature [e.g. Rosenthal & Lasnitzki, 1928]. We observed usually a high aerobic glycolysis of the same order as the respiration. Both activities showed a rather rapid fall.

Table II. *Metabolism of various glycolysing tissues with and without M/30 NH_4Cl. Bicarbonate-glucose-Ringer*

Species	Tissue	With NH_4Cl						Control					
		Q_{O_2}		$Q_G^{O_2}$		$Q_G^{N_2}$		Q_{O_2}		$Q_G^{O_2}$		$Q_G^{N_2}$	
		1st hr.	2nd hr	1st hr.	2nd hr.	1st hr.	2nd hr.	1st hr.	2nd hr.	1st hr.	2nd hr.	1st hr.	2nd hr.
Guinea-pig	Medulla of kidney	-10·2	-9·7	14·0	12·8	21·6	21·5	-6·8	-6·9	14·3	13·1	24·0	22·0
Guinea-pig	Retina	—	—	—	—	61·6	—	—	—	—	—	51·7	—
Rat	Testis	-11·3	-5·1	3·7	2·1	6·0	4·9	-7·5	-7·9	2·9	1·8	7·4	6·5
Rat	Spleen (a)	-17·5	—	6·8	—	9·0	—	-10·0	—	4·4	—	11·7	—
	(b)	-15·2	-15·4	4·5	7·7	5·0	5·4	-12·9	-12·7	4·2	4·2	7·8	7·3
Rat	Diaphragm	—	—	—	—	3·8	—	—	—	—	—	4·1	—
Rat	Jensen sarcoma	-18·2	-14·7	25·6	19·2	30·9	24·2	-14·4	-12·4	22·8	21·9	29·0	21·6
Mouse	Yolk sac*	—	—	—	—	14·8	10·6	—	—	—	—	24·6	22·4
Mouse	Embryo†	-8·9	-8·1	2·9	1·7	9·5	7·7	-10·4	-8·2	3·1	3·0	7·1	6·2
Rat	Intestinal mucosa (a)	1-10 min. -47·5	1-60 min. -25·0	1-10 min. 59·5	1-60 min. 33·6	1-10 min. 22·1	1-60 min. 14·4	1-10 min. -18·7	1-60 min. -16·4	1-10 min. 42·0	1-60 min. 24·2	1-10 min. 45·6	1-60 min. 25·0
	(b)	-26·7	-18·1	28·4	18·5	23·2	14·8	-24·0	-15·4	26·8	15·5	38·4	20·4
	(c)	-6·3	-3·7	12·9	6·3	—	13·2	-27·3	-13·4	32·2	15·8	—	20·5

* Almost full term. † Average dry wt. 8 mg.

reaches its maximum value after about 60 min. (Table III). Variation of the glutamic acid concentration between $M/50$ and $M/1000$ does not greatly affect the inhibition.

Table III. *Inhibition of anaerobic glycolysis of brain slices by l(+)glutamic acid*

Species	Conc.	With glutamic acid		Control		Inhibition %		Remarks
		1–10 min.	60–80 min.	1–10 min.	60–80 min.	1–10 min.	60–80 min.	
Rat	$M/100$	10·5	5·1	16·0	13·8	35	63	Mean of 4 exp.
Guinea-pig	$M/100$	12·2	6·0	21·0	18·4	42	67	Mean of 8 exp.
Rat	$M/1000$	13·5	5·4	15·6	12·1	13·5	55·5	
Guinea-pig	$M/1000$	18·2	8·5	18·9	14·8	4	42·5	Mean of 4 exp.

The effect seems to be specific for brain (Table IV). Even in retina it was found to be absent or, at the most, very feeble. Neither the anaerobic glycolysis of yeast cells, nor that of defibrinated blood (guinea-pig), nor the acid production from starch in a dialysed muscle extract (rabbit) was inhibited by glutamic acid.

Table IV. *Effect of l(+)glutamic acid on the anaerobic glycolysis of various glycolysing tissues*

Species	Tissue	$Q_C^{N_2}$ (1st hr.)	
		With $M/100$ glutamate	Control
Guinea-pig	Retina	48·7	51·8
Pigeon	Retina	76·0	81·0
Guinea-pig	Medulla of kidney	27·6	29·0
Rat	Intestinal mucosa	16·3	15·5
Guinea-pig	Intestine (whole wall)	7·5	6·9
Guinea-pig	Spleen	5·3	5·9
Rat	Testis	7·2	7·5
Rat	Diaphragm	6·1	5·9
Rat	Jensen sarcoma	36·9	39·2
Rat	Yolk sac	13·1	12·9
Guinea-pig	Heart	7·2	6·5

Specificity of the effect. A few closely related substances cause similar inhibition of the anaerobic glycolysis of brain tissue. They are: (1) the non-natural isomeride, $d(-)$glutamic acid; (2) glutamine; (3) dl-β-hydroxyglutamic acid[1] (Table V and VI). $d(-)$Glutamic acid is slightly less efficient than $l(+)$glutamic acid: at a concentration of $M/3000$ where the l-acid is still seen to cause some inhibition even a stimulating effect appears. The effects of the two stereoisomerides in different concentrations are compared in the same experiment (Table V). The actions of glutamine and hydroxyglutamic acid are shown in Table VI. Oxidized and reduced glutathione are also included, although they did not affect the anaerobic glycolysis; there was perhaps an initial activation by the reduced glutathione. The observation of Geiger [1935] that oxidized glutathione inhibits the anaerobic glycolysis of chopped brain could not be confirmed with brain slices. Geiger's results have been severely criticized by Dixon [1937].

Apart from the substances mentioned no other amino-acid of 13 tested[2] ($l(+)$alanine, $l(+)$valine, $l(-)$leucine, $l(-)$methionine, $l(-)$proline, $l(-)$hydroxy-

[1] Gift from Prof. Harington to Dr Krebs.
[2] Concentrations $M/100$. Retention was allowed for where necessary.

Table V. *Inhibition of anaerobic brain glycolysis by various concentrations of* l(+)- *and* d(−)*glutamic acids*

| | | With $l(+)$glutamic acid | | | | | With $d(-)$glutamic acid | | | |
| | | $Q_G^{N_2}$ | | % Inhibition | | | $Q_G^{N_2}$ | | % Inhibition | |
Species	Conc. 10^{-3} M	1–10 min.	60–80 min.	1–10 min.	60–80 min.	Conc. 10^{-3} M	1–10 min.	60–80 min.	1–10 min.	60–80 min.
Rat	0	15·6	12·1	—	—	0	15·6	12·1	—	—
	10	9·7	3·6	38	70	10	13·2	5·9	15	51
	3	10·9	3·9	30	68	3	11·9	5·8	23·5	52
	1	13·5	5·4	13·5	55	1	13·6	6·9	13	43
	0·3	16·9	9·4	0	22	0·3	20·1	12·7	0	0
Guinea-pig	0	19·6	15·4	—	—	0	19·6	15·4	—	—
	10	14·5	6·7	26	56·5	10	16·3	7·2	17	53
	3	15·2	7·6	22·5	51	3	16·9	11·5	14	25
	1	16·8	6·0	14	61	1	22·6	12·5	0	19
	0·3	17·0	12·2	13	21	0·3	23·5	18·9	0	0

Table VI. *Specificity of the inhibition of anaerobic brain glycolysis by glutamic acid and related substances*

| | | $Q_G^{N_2}$ | | % Inhibition | | |
Species	Addition	1–10 min.	60–80 min.	1–10 min.	60–80 min.	Remarks
Rat	0	16·7	14·8	—	—	Mean of 2 exp.
	$M/100$ $d(-)$glutamic acid	12·8	8·8	23	41	
Guinea-pig	0	21·1	17·9	—	—	Mean of 5 exp.
	$M/100$ $d(-)$glutamic acid	16·6	8·5	21	53	
Guinea-pig	0	19·3	17·7	—	—	
	$M/100$ $l(+)$glutamic acid	10·5	5·5	45	69	
	$M/100$ dl-β-hydroxyglutamic acid	13·6	6·8	30	62	
	$M/100$ glutamine	14·8	8·1	23	54	
	$M/500$ glutamine	18·8	14·1	3	20	
Rabbit	0	17·5	12·8	—	—	
	$M/100$ $d(-)$glutamic acid	12·0	7·1	31	45	
	$M/100$ glutamine	10·1	4·8	42	62	
Guinea-pig	0	18·6	17·0	—	—	
	$M/100$ glutathione (GSSG)	18·1	16·8	—	—	
Guinea-pig	0	14·0	10·1	—	—	Gas mixture freed from O_2 over hot copper
	$M/100$ glutathione (GSH)	19·7	11·8	—	—	

proline, dl-serine, $l(-)$aspartic acid, $l(-)$pyrrolidonecarboxylic acid, $l(+)$ornithine, $l(+)$arginine, $l(-)$histidine, $l(-)$tryptophan) affected the anaerobic glycolysis of brain slices. In addition a large number of mono-, di- and tri-basic organic acids (for some of them see Table XVI) were tested. Except for a slight unspecific inhibition in a few cases, no effect was found. The effect of the glutamic acid group is therefore quite specific.

(b) Augmentation of the glutamic acid effect on anaerobic glycolysis

Meyerhof & Lohmann [1926, 1, 2] found that lactic acid in $M/100$–$M/50$ concentration inhibited anaerobic glycolysis of rat brain by about 50 %. As in the case of glutamic acid they observed a similar effect of the natural and unnatural isomerides. Dickens & Greville [1933] who reinvestigated this effect found a similar inhibition of anaerobic brain glycolysis only with higher concentrations ($M/10$–$M/20$); they also found $d(-)$lactate less efficient than $l(+)$lactate.

$M/100$ dl-lactate did not inhibit the anaerobic glycolysis of guinea-pig brain in our experiments; in an experiment with rat brain however some inhibition was observed. $M/100$ lactate however always enhanced the inhibition caused by glutamic acid. This effect is especially large, when small concentrations of glutamic acid are used (Table VII).

Table VII. *Increase of the glutamic acid effect on anaerobic brain glycolysis*

		$Q_G^{N_2}$		% inhibition		
Species	Addition	1–10 min.	60–80 min.	1–10 min.	60–80 min.	Remarks
Guinea-pig	0	19·9	17·5	—	—	
	$M/100$ dl-lactate	18·9	16·3	5	7	
	$M/100$ $l(+)$glutamate	13·1	7·3	34	58	
	$M/100$ dl-lactate + $M/100$ $l(+)$glutamate	9·6	3·7	52	79	
Rat	0	18·3	10·3	—	—	
	$M/100$ dl-lactate	14·4	6·2	21	40	
	$M/1000$ $l(+)$glutamate	12·4	6·6	32	36	
	$M/100$ dl-lactate + $M/1000$ $l(+)$glutamate	7·8	3·1	57	70	
Guinea-pig	0	23·1	20·0	—	—	
	$M/100$ succinate	22·5	19·1	3	4	
	$M/100$ $l(+)$glutamate	14·8	7·0	36	65	Mean of 4 exp.
	$M/100$ succinate + $M/100$ $l(+)$glutamate	8·3	3·5	64	82·5	
Guinea-pig	0	24·2	21·2	—	—	
	$M/1600$ $l(+)$glutamate	21·6	14·7	11	31	
	$M/100$ succinate + $M/1600$ $l(+)$glutamate	19·6	9·6	19	55	
		1st hr.	2nd hr.	1st hr.	2nd hr.	
Guinea-pig	0	17·4	17·1	—	—	
	$M/100$ dl-lactate	19·8	16·3	0	5	
	$M/100$ succinate	16·8	15·6	3	9	
	$M/100$ $l(+)$glutamate	9·1	4·9	48	71	
	$M/100$ dl-lactate + $M/100$ $l(+)$glutamate	7·9	4·2	55	75·5	
	$M/100$ succinate + $M/100$ $l(+)$glutamate	7·2	2·6	59	85	

Another substance which unexpectedly increased the glutamic acid effect was found to be succinic acid. $M/100$ succinic acid alone never affected the anaerobic glycolysis. In combination with glutamic acid however the residue of the anaerobic glycolysis observed in presence of glutamic acid alone was again reduced by nearly 50 %. The effect can again best be demonstrated with small concentrations of glutamic acid (Table VII, Fig. 1).

Many other substances which were tried in combination with glutamic acid did not influence its action.

(c) *Reversibility of the inhibition of anaerobic glycolysis*

The glutamic acid inhibition of anaerobic glycolysis of brain slices can be reversed by pyruvic acid (Table VIII, Fig. 2). Variation of the pyruvic acid concentration between $M/100$ and $M/1000$ does not change the result. The fall of anaerobic glycolysis is not entirely prevented, but only delayed. After some time inhibition again prevails. Pyruvic acid also relieves the inhibition caused by $d(-)$glutamic acid and by the combination of glutamic + succinic acids. The

action of pyruvic acid is less efficient if it is added after the first 20 min. of the experiment.

A similar effect, though to a smaller extent, is displayed by α-ketoglutaric acid (Table VIII).
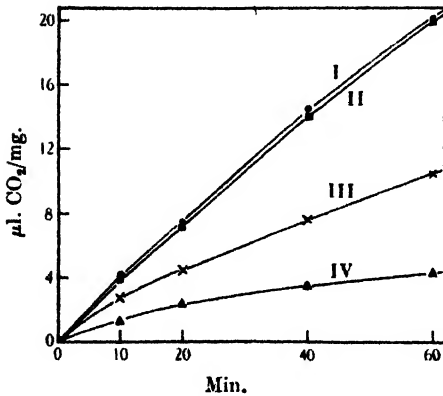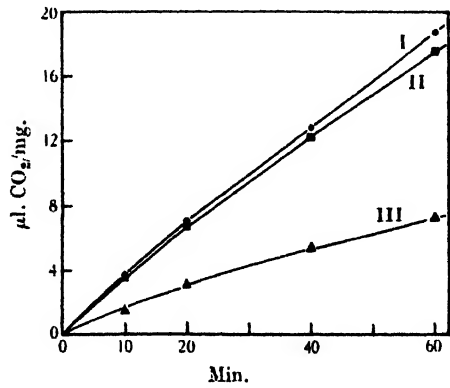
Fig. 1.   Fig. 2.

Fig. 1. Anaerobic glycolysis of guinea-pig brain slices in bicarbonate-glucose-Ringer. I, no addition; II, with $M/100$ succinate; III, with $M/100$ $l(+)$glutamate; IV, with $M/100$ succinate + $M/100$ $l(+)$glutamate.

Fig. 2. Anaerobic glycolysis of rat brain slices in bicarbonate-glucose-Ringer. I, with $M/100$ pyruvate; II, with $M/100$ pyruvate + $M/100$ $l(+)$glutamate; III, with $M/100$ $l(+)$glutamate.

Table VIII. *Reversal of the glutamic acid effect on anaerobic brain glycolysis*

Species	Addition	$Q_G^{N_2}$		% Inhibition	
		1–10 min.	60–80 min.	1–10 min.	60–80 min.
Rat	0	16·7	14·9	—	—
	$M/500$ pyruvate	21·7	17·8	—	—
	$M/100$ $l(+)$glutamate	9·1	6·0	45·5	60
	$M/100$ $l(+)$glutamate + $M/500$ pyruvate	21·6	15·3	0	0
Guinea-pig	0	23·2	19·4	—	—
	$M/100$ pyruvate	22·8	20·3	—	—
	$M/100$ $l(+)$glutamate	15·3	8·4	34·0	56·7
	$M/100$ $l(+)$glutamate + $M/100$ pyruvate	22·0	13·6	5	30
Guinea-pig	0	21·2	18·7	—	—
	$M/100$ $l(+)$glutamate + $M/100$ succinate	10·3	4·9	51·5	74
	$M/100$ $l(+)$glutamate + $M/100$ succinate + $M/100$ pyruvate	19·6	13·7	7·5	26·6
Guinea-pig	0	20·6	17·7	—	—
	$M/100$ $l(+)$glutamate + $M/100$ succinate { + $M/100$ pyruvate	17·5	13·4	15	24
	+ $M/300$ pyruvate	16·7	11·9	19	34
	+ $M/1000$ pyruvate	19·8	15·2	4	14
Guinea-pig	0	16·9	11·9	—	—
	$M/100$ $d(-)$glutamate	16·0	7·8	5·5	34·5
	$M/100$ $d(-)$glutamate + $M/100$ pyruvate	21·0	10·9	0	8
Guinea-pig	0	18·7	17·0	—	—
	$M/100$ α-ketoglutarate	23·2	21·0	—	—
	$M/100$ $l(+)$glutamate	14·3	7·4	23·5	56·5
	$M/100$ α-ketoglutarate + $M/100$ $l(+)$glutamate	18·2	11·4	3	33

As Mendel *et al.* [1931] have shown, pyruvic acid also counteracts the inhibition of anaerobic glycolysis of tumour tissue caused by glyceraldehyde. The same is true for embryonic tissue [Needham & Nowiński, 1937]. In brain however pyruvic acid cannot prevent the inhibition of anaerobic glycolysis by glyceraldehyde [Holmes, 1934; Baker, 1938].

The increased CO_2 evolution in presence of pyruvic acid is not due to an increased decarboxylation of pyruvic acid. The rate of anaerobic disappearance of pyruvic acid was determined by the carboxylase method after incubation with brain slices in presence and in absence of glucose and of $l(+)$glutamic acid. It was found to be independent of these (Table IX). A similar result has recently

Table IX. *Anaerobic disappearance of pyruvic acid after incubation with guinea-pig brain slices*

Initial concentration of pyruvic acid: $M/200$. Duration of exp.: 90 min.

Addition	$Q_{pyruvate}$ Exp. 1	Exp. 2
0	– 3·8	– 3·5
0·2 % glucose	· 4·4	– 3·0
0·2 % glucose + $M/100$ $l(+)$glutamate	– 4·3	– 3·2

been reported by Kritzmann [1938] who found that the disappearance of pyruvic acid from minced brain is not increased by the presence of $l(+)$glutamic acid. From a Q-value = 3–4 for pyruvic acid disappearance an extra CO_2 production of 1·5–2 (Q_{CO_2}) can be deduced [cf. Weil-Malherbe, 1937]. This is too small to account for the restoration of the acid production after inhibition by glutamic acid.

(d) Mechanism of the glutamic acid effect

(1) Since it is known that brain tissue can convert $l(+)$glutamic acid into α-ketoglutaric acid by oxidative deamination [Weil-Malherbe, 1936] the described effects might have been due to a liberation of NH_3 which indeed inhibits the anaerobic glycolysis of brain in fairly low concentrations, as has been shown in the first section of this paper. There are however several objections to this view: (*a*) the deamination is an oxidative process and does not occur anaerobically, at any rate not to any significant extent. (*b*) During the deamination of $l(+)$glutamic acid, owing to secondary reactions, the concentration of free NH_3 in the suspension medium does not rise. This has been well established for brain tissue under aerobic conditions [Krebs, 1935; Weil-Malherbe, 1936]. Table X

Table X. *Anaerobic ammonia formation by guinea-pig brain slices*

Addition (0·2 % glucose in all exp.)	$Q^{N_2}_{G}$ 1st hr.	$Q^{N_2}_{NH_3}$ 1st hr.
0	17·3	0·15
$M/100$ $l(+)$glutamate	11·4	0·11
$M/100$ $d(-)$glutamate	11·4	0·23
$M/100$ pyruvate	15·9	0·18
$M/100$ $l(+)$glutamate + $M/100$ pyruvate	15·9	0·21
$M/100$ $d(-)$glutamate + $M/100$ pyruvate	14·3	0·23

shows that there is no increase of free NH_3 in presence of $l(+)$- or $d(-)$glutamic acid under anaerobic conditions either. (*c*) $M/1000$ $l(+)$glutamic acid sometimes has a greater inhibitory effect than $M/1000$ NH_4Cl (Table XI).

Table XI. *Comparison of the inhibitions of anaerobic glycolysis of guinea-pig brain slices by M/1000 l(+)glutamate and M/1000 NH₄Cl*

	$Q_{G}^{N_2}$ (1st hr.)		
Addition	Exp. 1	Exp. 2	Exp. 3
0	13·3	15·4	16·8
M/1000 l(+)glutamate	10·2	9·5	13·7
M/1000 NH₄Cl	9·3	9·5	16·0

In spite of these facts it could be argued that a transitory liberation of a very small amount of NH_3 inside the cell may suffice to produce the same effects as a higher concentration in the surrounding medium. However, though a very slight deamination of l(+)glutamic acid cannot be excluded, this explanation cannot account for the effects of d(−)glutamic and β-hydroxyglutamic acids which are, as far as we know, not deaminated by brain tissue. The sample of d(−)glutamic acid used was probably optically pure. It was a gift from Dr Krebs and was prepared from the racemic acid by yeast fermentation. Analytical figures were published by Krebs [1935].

(2) Braunstein & Kritzmann [1937] suggested that the intermolecular transfer of amino groups, discovered by them, might, in presence of glutamic acid, lead to the formation of alanine instead of lactic acid from glucose and that this process might explain the inhibition of lactic acid formation by glutamic acid. This reaction would however involve the formation of an equivalent amount of α-ketoglutaric acid from glutamic acid, so that the rate of acid formation would remain the same and no inhibition would result in manometric experiments. It is of course possible that the "trapping" of the pyruvic acid arising during glycolysis deprives the glycolytic enzyme system of its natural hydrogen acceptor and that its replacement by the ketoglutaric acid formed from glutamic acid does not restore the original activity. The reversing effect of added pyruvate supports such an interpretation. But against it the following facts must be considered: (1) there is no increased consumption of pyruvate in presence of glutamic acid, at least as far as can be concluded from carboxylase estimations, (2) since d(−)glutamic and β-hydroxyglutamic acids act similarly to l(+)glutamic acid, one must assume that they can replace it in the process of amino transfer.

(3) l(+)Glutamic, d(−)glutamic and dl-β-hydroxyglutamic acids were the only substances found by Krebs [1935] to react with the enzyme concerned with the synthesis and hydrolysis of glutamine, the first as a natural substrate, the latter two causing competitive inhibition. Though there is as yet no other evidence for it, it is not impossible that the state of this enzyme, whether combined or free, has something to do with the control of glycolysis in nervous tissues. This would explain why the glutamic acid effect is specific for nervous tissue which is the only one of the highly glycolysing tissues where a synthesis of glutamine occurs.

(e) Action on aerobic glycolysis

Whereas the substances of the glutamic acid group depress the glycolysis of brain tissue under anaerobic conditions, they provoke on the other hand an appreciable aerobic lactic acid formation. Freshly cut brain slices, suspended in bicarbonate-glucose-Ringer solution have a fairly high aerobic glycolysis during the first 10–20 min. of the experiment ($Q_{G}^{O_2}$=4–6). Normally this aerobic glycolysis soon disappears entirely in the further course of the experiment. In the presence of the substances of the glutamic acid group (i.e. l(+)- and d(−)glutamic acids, glutamine and dl-β-hydroxyglutamic acid) it remains at

this high level. The respiration is unchanged or even accelerated, especially with $l(+)$glutamic acid. Reduced glutathione which differs from these substances in its action on anaerobic glycolysis has the same effect upon aerobic glycolysis of brain slices [see also Baker, 1937]. I confirmed Baker's finding that the autoxidation of reduced glutathione becomes quite negligible after a short incubation with brain slices. Nevertheless, only the results of the chemical lactic acid analysis are reproduced in Table XII.

Table XII. *Effect of glutamic acids on aerobic glycolysis of guinea-pig brain slices*

		$Q_G^{O_2}$		
Addition	Q_{O_2} 1st hr.	1–10 min.	60–80 min.	Remarks
0	− 10·3	3·7	1·0	Mean of 6 exp.
$M/100\ l(+)$glutamate	− 13·7	5·6	4·8	
0	− 10·7	3·8	1·3	Mean of 4 exp.
$M/100\ d(-)$glutamate	− 9·2	8·7	6·3	
0	− 9·9	4·5	1·7	
$M/100\ l$-glutamine	− 13·8	7·3	6·1	
0	− 10·3	3·0	0·6	
$M/100\ dl$-β-hydroxyglutamate	− 10·1	5·2	4·1	

	$Q_G^{O_2}$ (2 hr.)	
	Manometrically	Chemically
0	0·8	1·9
$M/100\ l(+)$glutamate	4·6	5·5
$M/100\ d(-)$glutamate	5·2	6·0
$M/100$ glutathione (GSH)	—	5·3

The rise of aerobic glycolysis is usually greater with the non-natural than with the natural glutamic acid.

In no other glycolysing tissue was an increase of aerobic glycolysis observed in presence of $l(+)$glutamic acid (Table XIII).

Table XIII. *Aerobic metabolism of various glycolysing tissues with glutamic acid*

Species	Tissue	With $M/100$ $l(+)$glutamate		Control	
		Q_{O_2}	$Q_G^{O_2}$	Q_{O_2}	$Q_G^{O_2}$
Rat	Testis	− 9·9	3·1	− 7·5	3·8
	Spleen	− 10·4	2·8	− 9·3	2·6
	Intestine (whole wall)	− 8·0	4·6	− 7·7	4·7
	Jensen sarcoma (a)	− 10·5	23·3	− 9·7	20·9
	(b)	− 12·7	26·1	− 12·3	30·8
	Yolk sac	− 4·4	1·9	− 2·5	3·9
Guinea-pig	Medulla of kidney	− 10·7	14·2	− 11·3	13·8

Pasteur-Meyerhof quotient: respiration, aerobic and anaerobic glycolyses of brain slices were simultaneously determined in several cases with both $l(+)$- and $d(-)$glutamic acids. Owing to the inhibition of the anaerobic glycolysis the aerobic glycolysis almost reaches the level of the anaerobic glycolysis. Thus the Pasteur-Meyerhof quotient $\left(\text{P.M.Q.} = \dfrac{Q_G^{N_2} - Q_G^{O_2}}{Q_{O_2}}\right)$ which was 1·8–2·0 in the controls fell to 0·2–0·6 in presence of glutamic acid. This is usually interpreted as an

indication that the Pasteur reaction is inhibited. But it is doubtful whether much significance can be attributed to these figures. We do not know whether or not the reactions which lead to an inhibition of glycolysis under anaerobic conditions also operate under aerobic conditions. Indeed, in the presence of high concentrations of NH_4^+ or K^+, the aerobic glycolysis rises to the level of the normal anaerobic glycolysis, while the anaerobic glycolysis is strongly inhibited in the same medium. This is obviously a case where aerobic and anaerobic conditions cannot be compared and where the P.M.Q. becomes meaningless. The same may be true for the effects of glutamic acid. As long as the aerobic glycolysis does not reach the level of the *normal* anaerobic glycolysis it is not safe to assume a breakdown of the Pasteur mechanism though, as in this case, the P.M.Q. may be practically zero.

III. The effect of maleic acid

Preliminary notes

(1) In several experiments solutions of maleic acid were prepared from freshly distilled maleic anhydride. The results differed in no way from those obtained with a sample of commercial maleic acid.

(2) Since the effects observed often change so quickly with time, the usual method of calculating average figures for respiration and glycolysis over periods of hours does not give an adequate impression of the real phenomena. The Q-values were therefore calculated for periods of 20 min. and the figures plotted at the midpoint of the corresponding period. Of course only a limited proportion of the experiments which were actually done can thus be communicated.

It will be noticed that $Q_{N_2}^{CO_2}$ often assumes negative values in the control experiment after some time. This is probably due to oxidative disappearance of acid initially formed.

(3) *Retention*: Solutions of $M/50$ maleic acid in bicarbonate-Ringer at pH 7·4 have a retention of about 9 %. In the early experiments no allowance for this was made, since it was believed that the smallness of the retention could not greatly influence the results and did not warrant the rather complicated extra analyses and calculations involved. Later however the retention was allowed for, using a new simplified principle developed by Dickens [unpublished]. It appeared that, if no allowance for retention were made, the curve of the aerobic glycolysis was quite similar to, and only slightly lower than the true glycolysis, corresponding to the proportion of the retention. The respiration, too, was hardly affected as long as the aerobic glycolysis was low. When the aerobic glycolysis was high, however, the errors became very serious indeed the respiration appearing too high by 100–150 %. Thus a secondary rise of respiration following the rise of aerobic glycolysis was revealed as spurious. With $M/50$ mixtures of glutamic and maleic acids or citric and maleic acids which have a retention of 12–14 % the errors amounted to several hundred per cent.

(a) The effect on brain metabolism

The action of maleic acid as a respiratory poison is well known [Thunberg, 1920; Grönvall, 1924; Gözsy & Szent-Györgyi, 1934]. I find that the inhibition of brain respiration depends (1) on the substrate present, (2) on the medium used: it is higher in phosphate than in bicarbonate medium.

(1) The inhibition in phosphate-Ringer solution is largest with glucose as substrate and only slightly less with lactate. It is on the other hand comparatively small with succinate and pyruvate, though it increases with time (Table XIV).

Table XIV. *Inhibition of brain respiration (guinea-pig) in phosphate-Ringer by M/50 maleate in presence of various substrates*

| | Q_{O_2} | | | | | | % Inhibition | | |
| | Without maleate | | | With maleate | | | 1st | 2nd | 3rd |
Substrate added	1st hr.	2nd hr.	3rd hr.	1st hr.	2nd hr.	3rd hr.	hr.	hr.	hr.
0·2 % glucose	− 9·8	− 10·0	− 10·3	− 4·3	− 1·2	− 0·8	56	88	92
M/50 lactate	−10·9	− 10·7	− 10·8	− 6·3	− 1·6	− 0·8	42	85	92
M/50 pyruvate	− 9·9	− 9·3	− 9·3	− 8·9	− 5·9	− 3·5	10	36	62
M/50 ketoglutarate	− 9·2	− 7·9	− 7·0	− 7·2	− 3·0	− 1·3	22	62	81
M/50 succinate	− 12·0	− 10·5	− 8·3	−11·3	− 6·9	− 4·2	6	34	49
M/50 fumarate	− 9·1	− 7·1	− 4·6	− 7·5	− 3·3	− 1·5	18	54	78
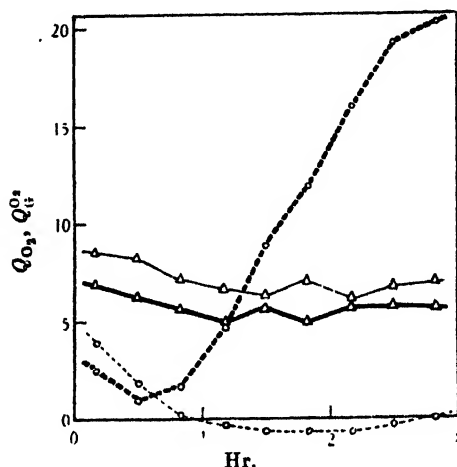
Fig. 3. Respiration and aerobic glycolysis of guinea-pig brain slices. Thick line: with $M/50$ maleate. Retention allowed for. Thin line: control in bicarbonate-glucose-Ringer. △- --△ respiration. o----o aerobic glycolysis.
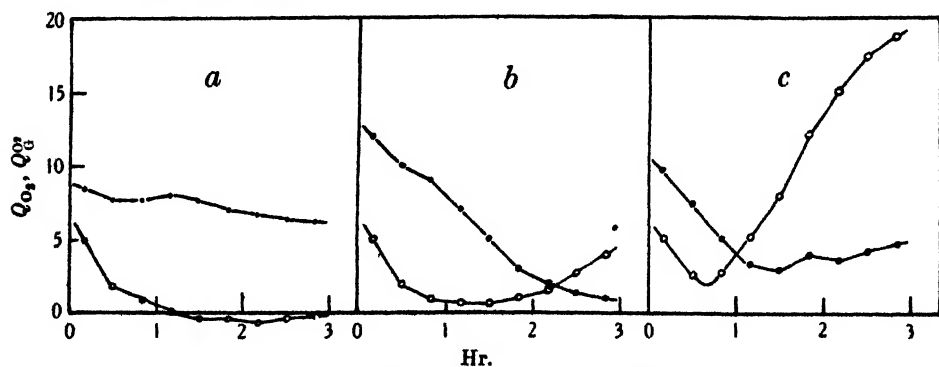
Fig. 4. Respiration and aerobic glycolysis of guinea-pig brain slices. (a) control; (b) with $M/100$ maleate; (c) with $M/50$ maleate. Retention allowed for. •——• respiration. o——o aerobic glycolysis.

(2) In bicarbonate-Ringer solution with glucose as substrate the following observations were made: in presence of $M/100$ maleate there is a slow and steady fall of respiration which at the end of the 3rd hr. reaches almost zero. The aerobic glycolysis does not differ much from the control; a slow rise is sometimes

observed in the 3rd hr. (Fig. 4). If the maleate concentration is increased to $M/50$, the fall of respiration is much less severe. In one experiment (Fig. 4) respiration fell to about 40–50 % of the control within the first 20 min. and remained at this level. In another experiment (Fig. 3) there was only about 20–30 % inhibition throughout. On the other hand there was a steady rise of the aerobic glycolysis starting after 30–60 min. and reaching the level of the normal anaerobic glycolysis towards the end of the 3rd hr. Values of $Q_0^n = 24$ have been observed (see Fig. 7). It seems that the onset of the strong aerobic glycolysis prevents the complete breakdown of respiration in presence of $M/50$ maleate. A concentration of $M/100$ maleate is too small to bring about an early and sufficiently large rise of aerobic glycolysis and therefore the paradoxical result is obtained that in this case the respiration is finally inhibited more strongly than with $M/50$ maleate.

In absence of glucose no acid formation occurs. Chemical analysis shows that the acid formed from glucose is lactic acid (Table XV).

Table XV. *Lactic acid formation (by chemical estimation)*
in presence of maleate. Guinea-pig brain

Addition	$Q_{lactate}$ mean of 3 hr.
0	2·5
$M/100$ maleate	5·3
$M/100$ maleate + $M/100$ glutamate	8·2
$M/100$ maleate + $M/100$ glutathione	9·6
0	2·7
$M/50$ maleate	7·2
$M/50$ maleate + $M/50$ glutamate	7·8
$M/50$ maleate + $M/50$ ketoglutarate	6·8

Specificity of the maleic acid effect. Though the action of maleic acid is not entirely a specific inhibition of the Pasteur reaction, it is of considerable interest for several reasons: (1) the inhibition of respiration is comparatively small; (2) maleic acid is a simple organic compound closely related to normal metabolites of the cell. Although there are several di- and tri-basic acids which are known to have toxic actions on cell metabolism, none of these caused such a drastic increase of aerobic glycolysis. The following acids have been tested: malonic, hydroxymalonic, hydroxymaleic, tartaric, racemic, dihydroxytartaric, oxalic, itaconic and citraconic acids (all in $M/50$ concentration). Oxalic acid, for instance, inhibited both respiration and glycolysis. Others, like hydroxymalonic acid, had an inhibitory action on brain respiration which was certainly not smaller than that of maleic acid. But although there was no inhibition of the anaerobic glycolysis,[1] the aerobic glycolysis was not significantly increased. $M/50$ maleic acid did not inhibit the anaerobic glycolysis of brain slices (Table XVI). This is in contrast to the experiments of Morgan & Friedmann [1938, 2] who, it is true, worked under different conditions since they used a concentration of $M/12·5$ and minced brain suspended in phosphate buffer.

It may be mentioned in this connexion that $M/50$ malonate does not affect the respiration of brain slices in bicarbonate-glucose-Ringer solution. In phosphate-glucose-Ringer there is an inhibition of 40–50% (Table XVI).

[1] Jowett & Quastel [1937] found an inhibition of anaerobic brain glycolysis with $M/14$ hydroxymalonate.

Table XVI. *Metabolism of guinea-pig brain slices in presence of dibasic acids (non-metabolites)*

B = Bicarbonate-glucose-Ringer; P = phosphate-glucose-Ringer.

Addition	Medium	Q_{O_2}			$Q_G^{O_2}$			$Q_G^{N_2}$		
		1st hr.	2nd hr.	3rd hr.	1st hr.	2nd hr.	3rd hr.	1st hr.	2nd hr.	3rd hr.
0	B	−7·8	−7·0	−7·0	3·7	0·5	0	17·9	14·6	—
M/60 oxalate*		−15·0	−7·1	−3·7	6·0	2·6	1·9	5·1	3·9	—
M/50 malonate		−8·4	−7·0	−6·9	1·9	0·9	1·6	12·8	10·0	—
M/50 hydroxymalonate		−7·8	−4·9	−5·4	0·2	1·5	1·1	17·7	14·5	—
0	B	−7·8	−7·1	—	1·7	−1·0	—	19·9	18·4	—
M/50 malonate		−9·4	−7·8	—	1·6	−0·7	—	19·5	18·3	—
0	P	−8·5	−8·6	—	—	—	—	—	—	—
M/50 malonate		−5·2	−4·1	—	—	—	—	—	—	—
0	B	—	—	—	—	—	—	20·5	18·6	—
M/50 maleate		—	—	—	—	—	—	19·9	18·4	—
M/50 hydroxymalonate		—	—	—	—	—	—	20·0	18·9	—
0	B	—	—	—	—	—	—	19·1	16·2	15·7
M/50 maleate		—	—	—	—	—	—	19·2	15·7	15·0

* (Ca++-content of Ringer solution allowed for.

Table XVII. *Metabolism of various glycolysing tissues in presence of M/50 maleic acid*

		With M/50 maleate								Control								
		Q_{O_2}			$Q_G^{O_2}$			$Q_G^{N_2}$		Q_{O_2}			$Q_G^{O_2}$			$Q_G^{N_2}$	
Species	Tissue	1st hr.	2nd hr.	3rd hr.	1st hr.	2nd hr.	3rd hr.	1st hr.	2nd hr.	1st hr.	2nd hr.	3rd hr.	1st hr.	2nd hr.	3rd hr.	1st hr.	2nd hr.
Guinea-pig	Medulla of kidney (a)	−5·6	−6·3	−2·9	10·2	11·5	10·7	24·0	23·0	−11·8	−11·0	−13·7	11·2	9·6	8·9	27·4	26·5
	(b)	−5·4	−5·5	−7·3	9·2	10·2	11·5	—	—	−10·8	−9·7	−8·5	12·2	11·1	9·7	—	—
Guinea-pig	Heart	−4·1	−1·8	—	4·5	5·2	—	4·4	3·2	−5·3	−2·9	—	2·4	1·4	—	6·5	4·4
Rat	Intestinal mucosa	−7·0	—	—	16·2	—	—	—	—	−13·3	—	—	17·5	—	—	—	—
Rat	Testis	−7·5	−4·5	—	2·1	1·5	—	—	—	−9·2	−7·1	—	2·0	0·4	—	—	—
Rat	Spleen (a)	−6·9	−7·1	—	5·4	8·8	—	—	—	−14·3	−13·7	—	3·3	4·8	—	—	—
	(b)	−7·7	−4·6	—	6·0	9·6	—	7·2	7·0	−12·9	−12·8	—	4·2	4·2	—	7·8	7·3
Rat	Jensen sarcoma (a)	−3·5	−0	—	32·1	27·7	—	30·4	21·9	−14·4	−12·4	—	19·9	19·4	—	29·0	21·6
	(b)	−9·0	−3·5	—	23·9	22·7	—	35·2	30·9	−7·1	−4·8	—	23·8	22·9	—	37·0	32·0
Rat	Embryo*	−8·9	−5·3	—	9·4	12·7	—	16·5	15·2	−11·1	−10·4	—	5·0	4·6	—	19·5	18·6

* Average dry wt. 5 mg.

Influence of metabolites on the maleic acid effect

Certain substrates seem to increase the toxicity of maleic acid. With $M/50$ $l(+)$glutamic acid there is an almost immediate rise of the aerobic glycolysis to the normal anaerobic level followed by a rapid fall. The respiration falls sharply from the beginning (Fig. 5a). $d(-)$Glutamic acid has the same effect. With $M/50$ citrate there is a similar fall of respiration. The aerobic glycolysis reaches its peak after 90 min. and declines later (Fig. 5b). The presence of $M/50$ pyruvate on the other hand not only keeps the respiration intact, but also abolishes the effect on aerobic glycolysis to a large extent (Fig. 5c).
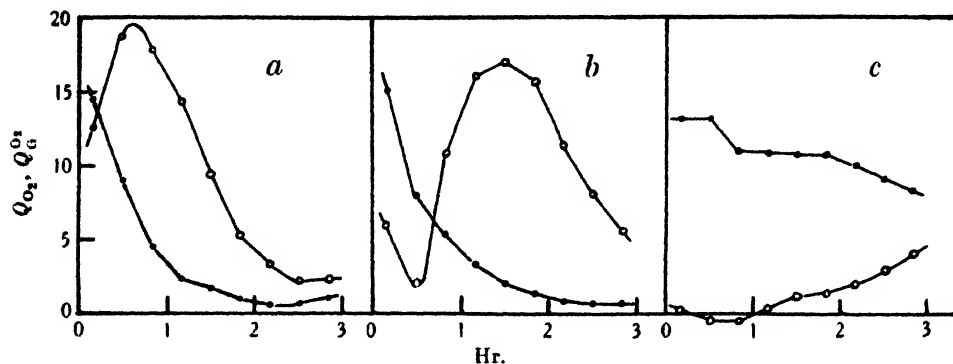
Fig. 5. Respiration and aerobic glycolysis of guinea-pig brain slices. (a) with $M/50$ maleate $+ M/50$ $l(+)$glutamate; (b) with $M/50$ maleate $+ M/50$ citrate; (c) with $M/50$ maleate $+ M/50$ pyruvate. Retention allowed for in all experiments. •——• respiration. o——o aerobic glycolysis.

In Fig. 6 some of the older experiments are reproduced. Since no allowance for retention was then made, only the curves of the aerobic glycolysis which is only slightly affected by the retention are given. $M/50$ ketoglutarate has an effect similar to that of citrate. With $M/50$ fumarate the aerobic glycolysis is diminished.

Although the aerobic glycolysis is hardly increased by $M/100$ maleate addition of $M/100$ glutamate raises it strongly, especially during the 2nd hr. $M/100$ reduced glutathione has a very similar effect (see also Table XV). This is particularly interesting in view of the reaction of maleic acid with thiol compounds discovered by Morgan & Friedmann [1938, 1]. If the effects of maleic acid were due to a destruction of the glutathione of the tissue, the addition of an equivalent amount of glutathione together with the maleic acid should neutralize the effect. What happens is actually the reverse: the effect of maleic acid is accentuated, as is the case with glutamic acid.

Another fact may here be commented upon: the anaerobic glycolysis of brain slices is inhibited by a combination of $M/100$ glutamic acid $+ M/100$ maleic acid to approximately the same extent as by $M/100$ glutamic acid alone. Yet under aerobic conditions a strong glycolysis occurs which may be double the glycolysis occurring in the same medium under anaerobic conditions. As pointed out before, the P.M.Q. is here meaningless.

Reversibility: the effect of maleic acid can to some extent be reversed by washing (Fig. 7). After 2 hr. incubation with $M/50$ maleate the slices were removed from the vessels and rinsed several times with Ringer solution; the manometric measurements were then resumed without maleate. In another pair of vessels the observation of the metabolism in presence of $M/50$ maleate was
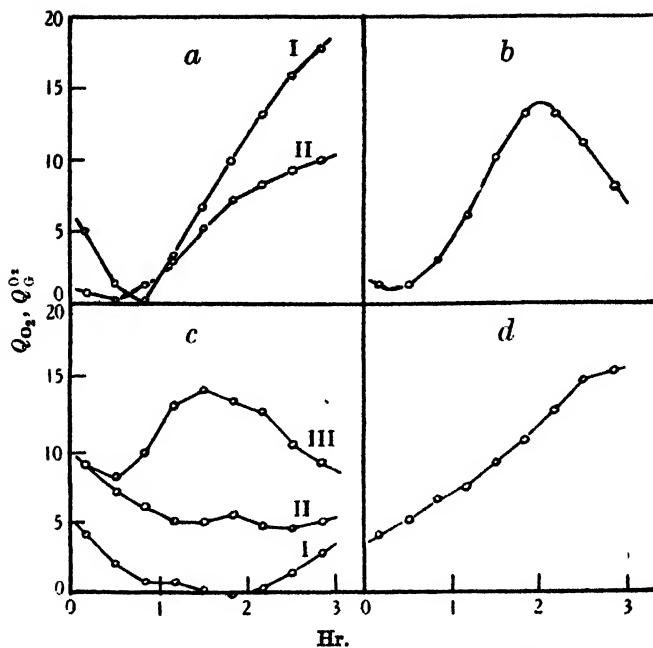
Fig. 6. Aerobic glycolysis of guinea-pig brain slices. (a) I, with $M/50$ maleate; II, with $M/50$ maleate + $M/50$ fumarate. (b) Same exp. $M/50$ maleate + $M/50$ α-ketoglutarate. (c) I, with $M/100$ maleate; II, with $M/100$ $l(+)$glutamate; III, with $M/100$ maleate + $M/100$ $l(+)$gluta-mate. (d) Same exp. $M/100$ maleate + $M/100$ GSH.
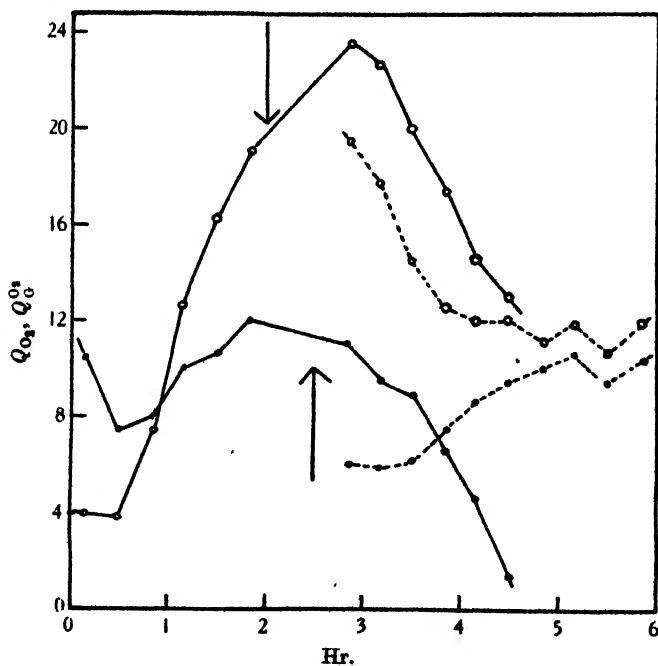
Fig. 7. Reversibility of the maleic acid effect by washing. Guinea-pig brain slices. Downward arrow: slices removed from $M/50$ maleate sol. Upward arrow: slices put back in thermostat after washing. Continuous line: metabolism in presence of $M/50$ maleate. The rise of respira-tion in the 2nd hr. is probably spurious, since no allowance for retention was made. Broken line: metabolism in bicarbonate-glucose-Ringer after washing. ●——● respiration. o——o aerobic glycolysis.

continued until its final destruction. There was, after the washing, some fall of aerobic glycolysis, but not to the normal level, while the respiration recovered almost completely. Another experiment gave a similar result.

(b) *The effect on other tissues*

The effect of maleic acid is not restricted to brain, but was also observed in other tissues, though to a less marked degree. The inhibition of respiration is usually higher in these tissues (Table XVII). A rise of aerobic glycolysis occurred for instance in embryo, heart and spleen, also in one experiment with Jensen sarcoma, where the respiration was strongly inhibited. Retention was not allowed for in these experiments, but since the aerobic glycolysis was in most cases small or moderate, the errors of respiration values will be small.

(c) *The mechanism of the maleic acid effect*

(1) The slow development of the maleic acid effect might have been due to poisoning of the "ammonia-binding mechanism" of brain [Weil-Malherbe, 1936] which would lead to an increase of the concentration of NH_4^+ in the tissue. This would also have explained why the effect was so much accelerated by the presence of glutamic acid which would have acted as NH_3 donator. Estimations of NH_3 however showed that no increase occurred after 3 hr. incubation of brain slices with $M/50$ maleate or with $M/50$ maleate + $M/50$ glutamate.

(2) It is of course tempting to seek a connexion between the biological action of maleic acid and the recently discovered reaction with thiol groups [Morgan & Friedmann, 1938, 1, 2]. Indeed Lehmann & Needham [1938] quote the results of Morgan & Friedmann as supporting the theory that the glycolysis of brain needs glutathione as coenzyme. Yet, as has been shown, glutathione does not reverse the action of maleic acid, but, on the contrary, enhances it.

Another compound which is known to react with thiol compounds is iodoacetic acid [Dickens, 1933]. But whereas maleic acid acted as a stimulant of glycolysis in the experiments described, iodoacetic acid is a very potent inhibitor of glycolysis. This action of iodoacetic acid too could not be correlated with the destruction of glutathione [Schroeder *et al.* 1933; cf. also Smythe, 1936]. Needham & Lehmann [1937] could not reverse the inhibition of glycolysis of embryonic tissue caused by iodoacetic acid by the addition of glutathione.

IV. RELEVANCE OF THE OBSERVED PHENOMENA TO THE THEORIES OF THE PASTEUR REACTION

Even if the action of a substance on isolated enzyme systems has been carefully studied, it may be dangerous to apply this knowledge directly to observations made on the intact cell, where its action may be quite different. As for maleic acid little is known about its action on isolated enzymes and nothing that could explain the observed effects. But that they are due to reactions with enzymes seems to be a justifiable conclusion in view of the inactivity of other substances with similar physical properties and a similar or even greater toxicity. This strengthens the case for an enzymic rather than a physico-chemical control of the Pasteur reaction.

Since *l*-glyceraldehyde is in all probability not an intermediate of animal metabolism [Needham & Lehmann, 1937], $l(+)$glutamic, $l(+)$lactic and succinic acids are the only naturally occurring substances which have been shown to inhibit anaerobic glycolysis. Their close connexion with the various

cycles of carbohydrate oxidation may suggest that the degree of saturation of certain dehydrogenases which are parts of these is a factor in the control of the Pasteur mechanism.

SUMMARY

1. NH_4^+ affects the metabolism of brain slices in a way similar to K^+, Rb^+ and Cs^+. $M/30$ NH_4Cl causes increase of respiration, increase of aerobic glycolysis to the anaerobic level and inhibition of anaerobic glycolysis. Some increase of aerobic glycolysis is still seen with concentrations of $M/1000$–$M/3000$ NH_4Cl. The only other tissue where a similar effect of $M/30$ NH_4Cl was observed was spleen. In intestinal mucosa the effect was observed once, but the observation could not be repeated.

2. $M/100$–$M/1000$ $l(+)$glutamic acid inhibits the anaerobic glycolysis of brain slices by 30–70%. $d(-)$Glutamic acid, l-glutamine and dl-β-hydroxy-glutamic acid act similarly. The effect is only observed in brain.

3. The effect of glutamic acid is increased by the addition of $M/100$ lactate or $M/100$ succinate.

4. The effect of glutamic acid is largely reversed by $M/100$–$M/1000$ pyruvate.

5. The substances of the glutamic acid group which inhibit the anaerobic glycolysis of brain increase the aerobic glycolysis to values of $Q_{0_2}^{N_2} = 5$–8. This effect, too, only occurs in brain.

6. $M/50$ maleic acid inhibits the respiration of brain slices in bicarbonate-glucose-Ringer to a degree varying in different experiments from 10 to 50%. Aerobic glycolysis rises slowly and reaches the anaerobic level during the 3rd hr. of the experiment. No other simple organic acid has a similar effect.

7. $M/50$ glutamate and $M/50$ citrate accelerate the effect of maleate; $M/50$ pyruvate abolishes it to a large extent.

8. Reduced glutathione does not reverse the effect of maleic acid, but like glutamic acid enhances it.

9. The effect of maleic acid can be partly reversed by washing.

10. In embryo, heart and spleen maleic acid causes a rise of aerobic glycolysis, which in some cases reaches the level of the anaerobic glycolysis.

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CCXC. THE FORMATION OF GLUCOSE FROM ACETOACETIC ACID IN RAT KIDNEY

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IN an earlier paper [Weil-Malherbe, 1938] the formation of a reducing substance from acetoacetic acid by rat kidney slices was reported. It will be shown that this substance is very probably glucose.

This observation is of interest in view of the still unsettled question of the transformation of fat into carbohydrate. Blixenkrone-Møller [1938] found an increase of liver glycogen after perfusion of cat liver with Na butyrate, but not with β-hydroxybutyrate. Haarmann & Schroeder [1938] described the formation of reducing substances from β-hydroxybutyric and dihydroxybutyric acids, but no evidence was brought forward by which these substances could be conclusively identified as carbohydrates. How important this identification is will be obvious in the light of the fact that, while the reducing compound formed by rat kidney slices from the first substance is glucose, it is mainly α-hydroxyacetoacetic acid or acetol in the second case.

Benoy & Elliott [1937] showed that a considerable increase of total carbohydrate is found after the aerobic incubation of rat kidney slices with pyruvate and, to a smaller extent, also with lactate, succinate or malate, though no synthesis of glycogen occurs in kidney.

EXPERIMENTAL

Estimation of reducing power. The reagent of Somogyi [1937] was used throughout. An empirical calibration curve for glucose was determined, which was almost linear except for very low concentrations, 1 ml. $M/100$ I_2 indicating $30\,\mu l.$ glucose. In the case of acetol 1 ml. indicates $112\,\mu l.$

If not stated otherwise, proteins and interfering substances were removed by $Cd(OH)_2$ precipitation [Miller & Van Slyke, 1936]. The reagent used contained $13 \cdot 0$ g. $3CdSO_4$, $8H_2O$ and $63 \cdot 5$ ml. N H_2SO_4 in 100 ml. $0 \cdot 8$ ml. of this solution $+ 1 \cdot 1$ ml. N NaOH were added per 10 ml.

The results are expressed in the conventional Q-units

$$\left(\text{e.g. } Q_{\text{glucose}} = \frac{\mu l.\ \text{glucose}}{\text{mg. dry wt.} \times \text{hr.}}\right).$$

I. *Formation of a reducing substance*

After aerobic incubation of rat kidney slices with acetoacetic acid an increased amount of a reducing substance is found in the suspension fluid. The magnitude of the effect may be seen from Table I where the results are calculated on the assumption that the reducing substance is glucose.

Table I. *Formation of reducing substance by slices of rat kidney cortex*

Bicarbonate-saline. $O_2 + 5\% CO_2$. 3 hr. incubation

Exp.	Substrate	$Q_{glucose}$
1	0	0·11
	$M/50$ acetoacetate	0·41
2	0	0·12
	$M/50$ acetoacetate	0·34
3	0	0·07
	$M/50$ acetoacetate	0·26

The amount of reducing substance slowly increases with time (Fig. 1).
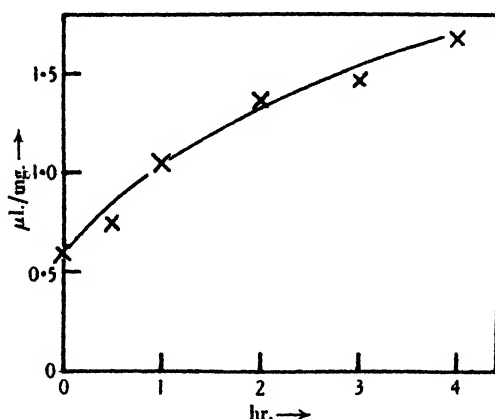
Fig. 1. Increase of reducing substance (calc. as glucose) with time.

No soluble reducing substances were found after aerobic incubation of acetoacetic acid with slices of brain, heart, Jensen rat sarcoma or with testicular tissue.

II. *Identification of the reducing substance as glucose*

(1) The reducing substance was found not to be volatile from neutral, acid or alkaline solution. The skatole test of Jordan & Pryde [1938] gave a vivid purple colour after heating to 80°, while it was negative at 40°, thus indicating the presence of an aldohexose.

Each of two vessels contained approx. 200 mg. dry wt. of slices suspended in 25 ml. bicarbonate-saline. The gas space was filled with 5% CO_2 in O_2. One of the vessels also contained $M/50$ aceto-acetate as substrate. After 3 hr. incubation the tissue was removed and 3 ml. $CdSO_4$ and 4 ml. N NaOH were added. The filtrate was neutralized and evaporated *in vacuo* to dryness. The residue was dissolved in 1 ml. water. 10 ml. conc. HCl and 10 mg. recryst. skatole were then added and the solutions put in a water bath at 37·5° for 30 min. No colour developed. After transfer to a water bath at 80° a deep violet colour soon developed in the solution with acetoacetic acid. The colour in the control appeared much later and was very much feebler. A pure solution of acetoacetate showed no colour at all.

(2) One of the most characteristic properties of glucose is its fermentability by yeast. Instead of estimating the reduction before and after fermentation, as is the usual procedure, the CO_2 of fermentation was measured manometrically thus preventing the possible loss of reducing substances by processes other than fermentation. The ratio of μl. glucose by reduction : μl. CO_2 of fermentation was

simultaneously determined in the experimental solutions and in a standard solution of glucose. The latter was made up with an amount of saline equal to that used for the experiment and was subsequently subjected to an identical treatment so that the final salt concentration in the standard and experimental solutions was the same. In addition a control solution without glucose containing an equal salt concentration was prepared. This was found necessary, as the blank gas evolution was somewhat increased in the high salt concentration. Also the yield of CO_2 of fermentation which was 90–92% in $M/30$ KH_2PO_4 (reduction/fermentation = 0·55) was slightly lower and varied from 82 to 90% (reduction/fermentation = 0·61–0·56) according to the method of deproteinization and the salt concentration which it entailed. The yield was highest after a simple kieselguhr filtration though in the experimental solutions the reduction value greatly exceeded the value calculated from the fermentation, especially in absence of the substrate (Table II). The lowest yield (82%) was obtained after a combined tungstic acid-Cd(OH)$_2$ deproteinization, but by this procedure most of the non-sugar reducing material appeared to be eliminated and the ratio of reduction/fermentation was almost identical in the experimental and standard solutions. This is strong evidence for the identity of the substance estimated in both cases.

Table II. *Formation of fermentable reducing substance by slices of rat kidney cortex*

Exp.	Substrate	Method of deproteinization	$Q_{glucose}$ calc. from CO_2 of fermentation	Reduction/ fermentation	Reduction/ fermentation in standard of pure glucose
1	0	Kieselguhr	0·04	2·18	} 0·56
	$M/50$ acetoacetate		0·23	0·78	
2	0	Cd(OH)$_2$	0·08	0·93	} 0·59
	$M/50$ acetoacetate		0·29	0·68	
3	0	Na$_2$WO$_4$ +	0·07	0·62	} 0·61
	$M/50$ acetoacetate	Cd(OH)$_2$	0·28	0·61	

Each of two vessels contained 130–160 mg. dry wt. of slices suspended in 25 ml. bicarbonate-saline in an atmosphere of O_2 + 5% CO_2. One of the vessels (vessel II) contained $M/50$ acetoacetate. Duration of the experiment: 3 hr.

Deproteinization. Exp. 1: 1 ml. 10% H_3PO_4 (10 vol. syrupy H_3PO_4 in 100 vol. solution) was added to vessel I and 1·5 ml. 10% H_3PO_4 to vessel II. After ½ hr. in a boiling water bath (see below) the solutions were brought to pH 5–6 by the addition of 1 ml. and 1·5 ml. N NaOH respectively, and filtered after addition of kieselguhr.

Exp. 2: 3 ml. CdSO$_4$ sol. and 4 ml. N NaOH added and centrifuged.

Exp. 3: 2·5 ml. N H$_2$SO$_4$ and 2·5 ml. 10% Na$_2$WO$_4$ were added and the sol. centrifuged after 10 min. 2 ml. CdSO$_4$ sol. and 4 ml. N NaOH were then added to the supernatant solution and it was again centrifuged.

The deproteinized solutions were placed in a boiling water bath for 30 min. to decompose excess acetoacetic acid. After cooling they were adjusted to pH 5–6 by the addition of 10% H_3PO_4 and evaporated to dryness *in vacuo*. The dry residue was taken up in 5 ml. water. 2 ml. were transferred to the manometer vessel for the measurement of fermentation, while the remainder was used for estimation of reducing power. The side bulb of the manometer vessel contained 0·5 ml. of a 10% suspension of well washed D.C.L. baker's yeast in $M/30$ KH_2PO_4. The gas space was filled with 5% CO_2 in N_2, freed from O_2 over hot copper. Control vessels containing salt solutions with and without a known amount of glucose were set up simultaneously. The measurement was carried out at 25°.

(3) Finally glucose was estimated by the method of Butkevič & Gajevskaja [1935] based on the colorimetric estimation of the osazone formed from glucose. The conditions worked out by these authors were rigorously adhered to, except that a glucose standard solution was used for the preparation of the colorimetric standard (Table III).

Table III. *Formation of osazone-yielding substance by rat kidney slices*

Substrate	$Q_{glucose}$ estimated by reduction	$Q_{glucose}$ estimated by osazone method
0	0·12	0·04
$M/50$ acetoacetate	0·30	0·21

The arrangement of the experiment was as described under (1). After $Cd(OH)_2$ precipitation acetoacetic acid was decomposed by boiling and acetone removed by evaporation *in vacuo* as under (2). The residue was taken up in 10 ml. water, of which 3 ml. were used for estimation of reducing power and the rest for osazone formation. No hydrazone was formed with a pure solution of acetoacetic acid treated in this way.

The experiments described show that, on incubation of rat kidney slices with acetoacetic acid, a soluble, reducing, fermentable and osazone-yielding substance is formed in amounts which are from 3 to 7 times larger than in the control. The relative increase is larger with the more specific fermentation and osazone methods than with the unspecific reduction method. Since the skatole test which at 80° is specific for aldohexoses was strongly positive, it can be safely assumed that the substance in question is indeed glucose.

III. *Increase of total fermentable carbohydrate*

It remained to decide whether the effect observed was due to a sparing action of acetoacetic acid on preformed carbohydrate or to a true synthesis of glucose. Analyses of the total fermentable carbohydrate showed a considerable increase after incubation with acetoacetic acid, thus indicating a synthesis (Table IV).

Table IV. *Increase of total fermentable carbohydrate in rat kidney slices*

μl. total fermentable carbohydrate (as glucose) per 100 mg. wet wt.

Exp.	With $M/50$ acetoacetate		Without substrate	
	Initial	Final	Initial	Final
1	9·2	14·5	8·5	8·4
2	8·1	21·8	9·2	8·0
3	10·0	23·3	8·0	6·5
4	8·8	14·5	7·4	15·4
5	8·8	17·6	6·6	12·2

Slices from 6 rat kidneys were distributed into 4 tared, stoppered weighing bottles containing saline and weighed. They were then transferred to vessels 1–4 (about 500 mg. slices (wet wt.) per vessel).

Vessel ...	1	2	3	4
Bicarb. saline (ml.)	8	8	8	8
0·4 M acetoacetate (ml.)	0	0	0·4	0·4
Addition of 0·5 ml. 10 N H_2SO_4	t_0	3 hr.	t_0	3 hr.

Gas space: 5% CO_2 in O_2.

After the experiment the vessels were put in a boiling water bath for 10 min. The contents were then transferred to a mortar, thoroughly ground and finally poured into a large test tube.

Vessel and mortar were rinsed 3 times with 1 ml. N H_2SO_4. The test tube was heated in a boiling water bath for 3 hr. After cooling, 2·5 ml. 10% NaOH and 1·3 ml. 10% sodium tungstate were added, the volume measured and the solution centrifuged. 1 ml. $CdSO_4$ sol. and 0·4 ml. 20% NaOH were added to the measured supernatant fluid and it was again centrifuged. The clear solution was made up to 10 ml. or, if more than 10 ml., its volume noted. The estimation of reducing power was carried out with 5 ml. before, and with 5 ml. after fermentation. For fermentation 5 ml. of a 10% suspension of well washed baker's yeast were centrifuged and the solution to be fermented was added to the residue. After thorough mixing the tube was left at room temp. for 15 min. before it was again centrifuged and the reduction determined in the supernatant solution.

IV. *The nature of the reducing substance formed from dihydroxybutyric acid by rat kidney slices*

Previously [Weil-Malherbe, 1938] experiments with rat kidney slices were described showing the disappearance of dihydroxybutyric acid and the simultaneous formation of a reducing compound. The latter was assumed to be largely acetol, since the intensity of the fluorescence test with o-aminobenzaldehyde roughly corresponded to that obtained with a solution containing an amount of acetol equivalent to the reduction value of the experimental solution. It was however desirable, in view of the results obtained with acetoacetic acid, to adduce further evidence and to decide whether glucose was formed besides acetol and if so in what quantity. The reducing power was therefore determined in the experimental solution before and after evaporation to dryness *in vacuo*, and the soluble fermentable carbohydrate in the non-volatile residue. The same analytical procedure was carried out with a control (no substrate) and with an experiment with acetoacetic acid as substrate. The decrease of reducing power after evaporation was calculated as acetol, that after fermentation as glucose.

Table V. *Formation of acetol and glucose by rat kidney slices*

Exp.	Substrate	Q_{acetol}	$Q_{glucose}$
1	0	0	0·01
	$M/50$ dl-threo-1:2-dihydroxybutyrate	2·03	0·12
	$M/50$ acetoacetate	0	0·23
2	0	0	0·14
	$M/50$ dl-threo-1:2-dihydroxybutyrate	2·3	0·11
	$M/50$ acetoacetate	0·07 (?)	0·31

Each of three vessels contained about 80 mg. slices (dry wt.) suspended in 8 ml. bicarbonate-saline. There was no substrate in the first, $M/50$ dl-threo-1:2-dihydroxybutyrate in the second and $M/50$ acetoacetate in the third vessel. The gas space was filled with 5% CO_2 in O_2. After an incubation of 2¼ hr. the solution was acidified with 0·3 ml. 2 N HCl, the tissue was removed and the solution neutralized to litmus with about 0·33 ml. N NaOH. A little kieselguhr was added and the solution was heated in a boiling water bath for 5 min. The clear filtrate was made up to 10 ml.: 3 ml. were taken for the estimation of reducing power immediately and the remaining 7 ml. were evaporated *in vacuo* to dryness. 2 ml. water were added to the dry residue, again evaporated and this process was repeated once more. The residue was taken up in 10 ml. water: 5 ml. were used for reduction before, and 5 ml. after fermentation, as described. A large proportion of the volatile reducing substance was recovered in the distillate from the solution containing dihydroxybutyric acid, whereas the distillates from the other two solutions did not contain any reducing substance.

It is quite clear from Table V that the results fully confirm the earlier observations and conclusions. A considerable quantity of a volatile reducing substance is formed in the experiment with dihydroxybutyric acid, but none in the control or in the acetoacetate experiment. The small amount of "acetol" found in Exp. 2 in the presence of acetoacetic acid is within the experimental error and is therefore insignificant. On the other hand much more soluble

fermentable carbohydrate is found in the presence of acetoacetic acid than in the other experiments. As far as the formation of glucose from dihydroxybutyric acid is concerned, there was no increase over the control in Exp. 2. Almost the same value as in Exp. 2 was observed in Exp. 1, but here the amount found in the control was unusually low and the apparent increase may well have been due to a sparing action.

There is therefore hardly any doubt that the metabolism of dihydroxybutyric acid and that of acetoacetic acid follow different routes and our earlier conclusion that neither dihydroxybutyric nor hydroxyacetoacetic acid is an intermediary in the oxidation of acetoacetic acid is confirmed.

V. *The mechanism of the formation of glucose from acetoacetic acid*

Benoy & Elliott [1937] find that the greatest synthesis of carbohydrate in rat kidney is observed in presence of pyruvic acid, and they conclude that pyruvic acid is an intermediary in the process of carbohydrate synthesis from other substrates, such as lactic, succinic and malic acids and alanine, all of which are known to be convertible into pyruvic acid.

The quantities of soluble reducing matter (calculated as glucose) found in the suspension fluid after 2 hr. incubation of rat kidney slices in presence and in absence of $l(+)$-alanine, acetoacetic acid and pyruvic acid are presented in Fig. 2, showing the enormous superiority of pyruvic acid. Though the conversion of acetoacetic acid into pyruvic acid has not yet been established, such a reaction may well occur. It is however extremely improbable that α-hydroxyacetoacetic acid is an intermediary in the process.

Should the oxidative conversion of acetoacetic into pyruvic acid be confirmed, it would mean another example of a cyclic oxidation of pyruvic acid, since pyruvic acid is converted into acetoacetic acid in many tissues (cf. Embden & Oppenheimer [1912], Annau [1934] and Edson [1935] for liver; Annau [1935] for liver, muscle and kidney; Weil-Malherbe [1936] for brain; Krebs & Johnson [1937] for muscle, heart, liver and kidney).
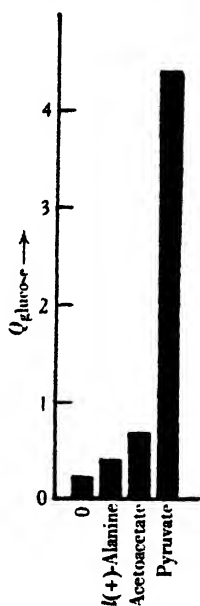
Fig. 2. Formation of reducing substance (cal. as glucose) from various substrates.

SUMMARY

If slices of rat kidney cortex are incubated with acetoacetic acid aerobically, an increased quantity of a reducing substance appears in the suspension fluid. This substance has been identified as glucose by the colour reaction with skatole, by its fermentability by yeast and by the formation of an osazone. An increase of the total fermentable carbohydrate indicates a true synthesis of carbohydrate from acetoacetic acid.

It is confirmed that no acetol is formed from acetoacetic acid, whereas the reducing substance formed from dihydroxybutyric acid is largely acetol, only a minute fraction being glucose.

It is suggested that pyruvic acid is an intermediary in the synthesis of glucose from acetoacetic acid.

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CCXCI. THE BIOCHEMISTRY OF SILICIC ACID

VII. DEPRESSION OF THE APPARENT SOLUBILITY OF SILICA IN BODY FLUIDS

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THE depression of solubility of SiO_2 through the agency of various metals and metallic oxides, notably Fe and Al, was briefly referred to in a former communication [King & McGeorge, 1938]. It was suggested that the apparent decrease in solubility of quartz and amorphous SiO_2 was due to precipitation of the dissolved silicic acid as the insoluble silicate of the metal. The phenomenon has now been explored further, and the effects of various mineral dusts on the solution of SiO_2 from quartz have been investigated.

Most of the mineral silicates are much less soluble in blood serum and ascitic fluid than are the free forms of SiO_2. Kaolin is particularly insoluble. In a mixture of kaolin and quartz powder suspended in a liquid it might be expected that the amount of SiO_2 dissolved would be conditioned by the most active member of the mixture, and that in the presence of an excess of the mixed powder the solubility would approach that of quartz powder taken alone. Naturally occurring kaolins, such as those represented in shales, contain variable, sometimes large, amounts of free SiO_2, and yet these natural stone dusts have an exceedingly low solubility. The amount of free SiO_2 represented in a sample of stone dust would lead to much more solution of silica if suspended alone in the same volume of liquid. This fact was brought out in the previous communication where it was shown that a stone dust (shale—used for mine dusting) had a solubility of less than 1 mg./100 ml. although it contained about 40 % free SiO_2, whereas the 0·8 g. free SiO_2 present in the 2 g. stone dust taken for 100 ml. ascitic fluid would if taken separately dissolve to the extent of 9 mg./100 ml. The figures set out in Table I demonstrate these points more fully. The methods used in this investigation—preparation and examination of dusts, solution experiments, separation of solid from liquid phase, determination of dissolved SiO_2 etc.—were those described in the previous communication [King & McGeorge, 1938]. The silicate minerals are much less soluble than quartz in the ascitic fluid used,[1] and the stone dusts are only slightly soluble despite their free SiO_2 contents which in some cases are large. The SiO_2 dissolved from the coal and coal-mine dusts is, of course, related to the silicate minerals contained in them, and is of the same order as that from the stone dusts.

The apparent depression of the solution of free SiO_2 contained in the stone dusts suggested that the effect might be extended to quartz admixed with the stone dust. This proved to be the case as is shown in Table II. The addition of increasing amounts of stone dust to 2 g. quartz suspended in 100 ml. ascitic fluid led to a progressive decrease in the SiO_2 dissolved, from 7 mg. SiO_2/100 ml. to

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[1] This ascitic fluid was of a more alkaline reaction (pH 8·0) than that used in the previous investigation, and the figures for solubility of the various dusts are hence not identical with those recorded in the previous paper.

Table I. *Dissolution of silica from mineral dusts*

2 g. dust suspended in 100 ml. ascitic fluid at 37°

Mg. SiO$_2$ per 100 ml.

Mineral dusts:	1 day	3 days	6 days
Quartz	4·4	5·4	7·0
Sericite	—	1·7	1·6
Chlorite (Mass.)	—	1·1	1·4
Chlorite (Saxony)	—	1·0	1·2
Kaolin	0·1	0·1	0·1
Biotite	—	1·0	1·1
Stone dusts:			
D. and C., S. 194* (39% free SiO$_2$, 10·8% Al)	0·5	0·6	0·6
C. and W., S. 20* (7% free SiO$_2$, 13·5% Al)	0·6	0·9	1·5
P. and P., S. 31* (21% free SiO$_2$, 9·1% Al)	1·4	1·5	1·3
Somerset Greys† (49% free SiO$_2$, 7·2% Al)	2·2	2·1	2·0
Pennant Rock† (42% free SiO$_2$, 5·9% Al)	2·4	2·5	2·2
Air-borne coal-mine dusts:			
Am. D. (16% ash)	1·2	1·4	1·4
Am. E. (39% ash)	1·5	1·4	1·1
Am. 1. (18% ash)	1·2	1·4	1·8
Am. J. (25% ash)	0·8	1·3	1·4
Coal dusts:			
Am. anthracite (3·9% ash)	0·9	1·5	2·0
Cy. bottom coal (2·5% ash)	1·5	2·1	2·1
Ba. soft coal (4·5% ash)	1·7	1·6	1·8
Yn. anthracite (6·3% ash)	1·5	1·2	1·4

* Shale dusts used for dusting underground.
† Coal-measure sandstones, produced by drilling.

Table II. *Dissolution of silica from mixtures of quartz and mineral dusts*

mg. SiO$_2$ dissolved by 100 ml. ascitic fluid

		1 day	3 days	6 days
2%*	Quartz (alone)	4·4	5·4	7·0
2%	D. and C. stone dust (alone)	0·5	0·6	0·6
2%	Quartz + 1% stone dust	3·2	3·4	4·6
2%	Quartz + 2% stone dust	1·8	1·7	2·4
2%	Quartz + 4% stone dust	1·6	1·4	1·7
2%	Yn. coal dust (alone)	1·5	1·2	1·4
2%	Quartz + 2% coal dust	2·9	2·6	3·7
2%	Quartz + 4% coal dust	2·2	1·8	2·2
2%	Kaolin (alone)	0·1	0·1	0·1
2%	Quartz + 2% kaolin	2·7	4·6	4·5
2%	Quartz + 4% kaolin	1·8	2·3	2·4
2%	Quartz + 1% Al$_2$O$_3$	0·4	0·3	0·3

* The % figures (e.g. 2% quartz) denote the number of g. dust per 100 ml. ascitic fluid.

1·7 mg. when twice as much stone dust as quartz was present. The phenomenon, moreover, seemed to be a general one, as a similar depression was obtained with several stone dusts and silicate minerals (Table III).

Coal dust, and the air-borne dusts from coal mines, appear to depress SiO$_2$ solubility in proportion to their silicate mineral content. Pure carbon (Kahlbaum's "carbo activ") and charcoal of low ash content have no effect on the solution of quartz. The depressions seen in Table IV would seem, therefore, to depend on the presence of stone dust in the coal. One of the coal dusts (Cy.) differs from the others in that it causes an increase in the amount of SiO$_2$ dissolved. This dust probably differs also from the others in the nature of its siliceous constituents.

Table III. *Effect of mineral dusts on the dissolution of quartz*

% reduction in amount of SiO_2 dissolved from 2 g. quartz in 100 ml. of ascitic fluid

	1 day	3 days	6 days
Sericite (2 g.)	—	49	61
Biotite (2 g.)	—	79	81
Chlorite (Mass.) (2 g.)	—	80	78
Chlorite (Saxony) (2 g.)	—	81	81
Kaolin (1 g.)	3	0	18
Kaolin (4 g.)	59	62	64
Al_2O_3 (1 g.)	90	96	96
Al_2O_3 (4 g.)	96	98	97
D. and C. stone dust (1 g.)	28	38	27
D. and C. stone dust (2 g.)	58	69	66
C. and W. stone dust (1 g.)	28	20	32
C. and W. stone dust (2 g.)	39	38	40
P. and P. stone dust (1 g.)	44	37	50
P. and P. stone dust (2 g.)	55	61	64
Somerset Greys (1 g.)	23	36	37
Somerset Greys (2 g.)	28	44	57
Pennant Rock (1 g.)	37	44	56
Pennant Rock (2 g.)	42	44	65

Table IV. *Effect of coal dust on the dissolution of quartz*

2 g. quartz, 4 g. coal dust per 100 ml. ascitic fluid; % reduction in amount of SiO_2 dissolved

	1 day %	3 days %	6 days %
Am. D. air-borne dust (16% ash)	23	6	21
Am. E. air-borne dust (39% ash)	53	63	75
Am. I. air-borne dust (18% ash)	33	50	54
Am. J. air-borne dust (25% ash)	71	64	71
Am. anthracite (3·9% ash)	40	30	35
Ba. soft coal (4·5% ash)	54	55	62
Yn. anthracite (6·3% ash)	51	69	69
Cy. bottom coal (2·5% ash)	(11)	(34)	(5% increase)

Depression of solubility of SiO_2 by these dusts may be dependent on adsorption of the dissolving silicic acid, on its precipitation as an insoluble silicate, or on the coating of the silica particles by some constituent of the depressor. In Table V are shown the results of adding silicate and mixed stone dusts to a saturated solution of SiO_2 in ascitic fluid which had been previously filtered free of all suspended particles. The depression of the amount of dissolved SiO_2 by the various mineral dusts is here clearly a case of precipitation or of adsorption. The SiO_2 dissolved in the liquid may react with some constituent of the mineral dust to form an insoluble precipitate. The coating of the solid particles of SiO_2 by a layer of the "depressor", e.g. of $Al(OH)_3$, would account for the depression of solubility shown in Table III, but this sort of mechanism cannot furnish a satisfactory explanation of the precipitation of dissolved SiO_2 shown in Table V. Further work on the composition of the solid phase of these mixtures should reveal an enrichment of the dust in SiO_2, and may possibly reveal the nature of the change taking place. Most mineral silicates are not of rigidly uniform composition, and it is possible that many of them may be capable of reacting with excess free SiO_2 with a consequent formation of a complex of higher SiO_2 content than the original.

The precipitation of SiO_2 from its solution by dusts from coal mines is shown in Table V. The results are somewhat irregular. No well-defined relationship is seen between the amount of depression of the SiO_2 in solution and the ash content. But the precipitation of dissolved SiO_2 has nevertheless almost certainly been

Table V. *Precipitation of dissolved silica by mineral dusts*

Decrease of dissolved SiO_2 in a "saturated solution" of quartz in ascitic fluid, 9 mg./100 ml., due to added dust

	g. dust added per 100 ml.	% decrease in dissolved SiO_2		
		1 day	3 days	6 days
Sericite	2	—	24	17
Biotite	2	—	33	35
Chlorite (Mass.)	2	—	76	75
Chlorite (Saxony)	2	—	69	66
Kaolin	2	32	29	26
	4	38	26	32
Al_2O_3	2	94	94	94
	4	95	95	96
D. and C. stone dust	2	24	31	38
	4	36	44	54
C. and W. stone dust	2	40	53	—
	4	46	50	—
P. and P. stone dust	2	27	25	18
	4	37	21	27
Somerset Greys	2	11	10	24
	4	22	20	30
Pennant Rock	2	8	5	12
	4	7	14	30
Am. D. air-borne dust	2	6	1	0
	4	9	4	4
Am. E. air-borne dust	2	4	21	20
	4	14	18	21
Am. I. air-borne dust	2	8	12	—
	4	16	23	12
Am. J. air-borne dust	2	20	—	7
	4	27	21	21

brought about by the minerals contaminating the coal. It is possible that adsorption of silicic acid by the carbonaceous particles of coal dust may play a part in the retardation of solution of SiO_2 and in the precipitation of SiO_2 already existing in solution, but this is unlikely since activated charcoal is without effect. The carbonaceous constituents of the coal may, however, affect the solubility of SiO_2 in an indirect manner, through an interaction (e.g. by adsorption) with some of the constituents of the ascitic fluid. Whether an adsorptive effect plays a part in the phenomenon of depression by coal dust of SiO_2 solubility, as well as the possibility of a reaction of the dissolved SiO_2 with different constituents of the dust will form the subject of further investigation.

The stone dusts used in this study[1] were samples of dust which have long been used for the stone-dusting of coal mines in Great Britain with the purpose of diminishing the hazard of explosion. Industrial experience during many years has led to the belief that stone-dusting in coal mines has not introduced or increased the silicosis hazard. Indeed the belief has slowly evolved that stone-

[1] The stone dusts were furnished by Dr A. G. R. Whitehouse, of the Safety in Mines Research Laboratory, who finds them to have a depressing action on the solution of free SiO_2 in Na_2CO_3 solution [1938], and with whom the authors have been fortunate to be in collaboration. The mineral dusts—sericite, chlorite, biotite and kaolin—were kindly given us by Dr A. Brammall, and the air-borne coal-mine dusts by Prof. H. V. A. Briscoe.

dusting actually diminishes the risk of silicosis in mines [cf. Haldane, 1917; 1931], and that certain mineral silicates may off-set the action of quartz in its relation to the disease. Recent experimental work on the effect of mineral dusts on the lungs and other tissues of animals [Gardner, 1938] has appeared to indicate that many mineral silicates may modify and even retard the action of free SiO_2. The finding that certain stone dusts modify the silicosis hazard may have its explanation in the experimental findings reported here, but at the present time it can only be interpreted as an interesting parallel which merits further study.

SUMMARY

The presence of shale and of some other silicate minerals may markedly diminish the amount of SiO_2 dissolved from a mixed rock dust in ascitic fluid. The amount of SiO_2 dissolved from quartz is reduced, and SiO_2 is removed from solution in the presence of these silicates.

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CCXCII. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS

LIX. SPINULOSIN (3:6-DIHYDROXY-4-METHOXY-2:5-TOLUQUINONE) A METABOLIC PRODUCT OF A STRAIN OF *ASPERGILLUS FUMIGATUS* FRESENIUS

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ANSLOW & RAISTRICK [1938, 1] reported the isolation, from cultures of a strain of *Aspergillus fumigatus* Fresenius grown on Raulin-Thom medium at 24°, of fumigatin, which they showed to be 3-hydroxy-4-methoxy-2:5-toluquinone. We have now shown that a different strain of *A. fumigatus*, when grown under the same cultural conditions, produces 6-hydroxyfumigatin, i.e. spinulosin (3:6-dihydroxy-4-methoxy-2:5-toluquinone). Spinulosin was first reported by Birkinshaw & Raistrick [1931] as a metabolic product of *Penicillium spinulosum* Thom and its constitution was settled by synthesis by Anslow & Raistrick [1938, 2].

The formation of spinulosin by *A. fumigatus* and *P. spinulosum* adds one more to the small but growing number of examples of species of moulds in different genera which give rise to the same metabolic product. The formation of fumigatin by one strain and of spinulosin by a different strain of the same species of mould is of interest mycologically since, if, as we believe, fumigatin and spinulosin function as oxidation-reduction systems in the vital processes of the moulds which produce them, then their formation points to quite different "oxidation levels" in two authentic strains of the same species of mould. It is of interest to note in this connexion that Anslow & Raistrick [1938, 1] found that only one of six strains of *A. fumigatus* examined gave fumigatin and none produced spinulosin.

EXPERIMENTAL

Culture

The culture used was, morphologically, undoubtedly a strain of *Aspergillus fumigatus* Fresenius. It was isolated by Mr G. Smith in October 1936 from dried Kentish hops, and bears the L.S.H.T.M. Catalogue number A 49. Thom & Church [1926], in their diagnosis of *A. fumigatus*, give "Colonies on Czapek's solution agar in some strains strictly velvety, in others with varying amounts of tufted aerial mycelium up to felted floccose forms." It is interesting to note that colonies of *A. fumigatus* A 49 are almost strictly velvety, sporing readily, whilst *A. fumigatus* A 46, the strain used by Anslow & Raistrick [1938, 1], for the production of fumigatin, gives colonies which are densely floccose, sporing very tardily. The two strains thus represent almost the extreme morphological range of this species.

Cultural conditions

The culture medium used was a Raulin-Thom solution of the following composition: glucose, 75 g.; tartaric acid, 4·0 g.; ammonium tartrate, 4·0 g.; $(NH_4)_2HPO_4$, 0·6 g.; K_2CO_3, 0·6 g.; $MgCO_3$, 0·4 g.; $(NH_4)_2SO_4$, 0·25 g.; $ZnSO_4$, $7H_2O$, 0·07 g.; $FeSO_4$, $7H_2O$, 0·07 g.; distilled water to 1500 ml. This medium was distributed in 350 ml. amounts in each of 100 1l. conical flasks, sterilized, sown with a spore suspension of *A. fumigatus*, A. 49, and incubated in the dark at 24°.

At the end of the incubation period (25–26 days) the flasks were uniform in appearance, having a crumpled grey mycelium with dark green sporing patches and a grey reverse. The metabolism solution, which was dark purple-red in colour, contained only 0·04 % of residual glucose (by polarimeter) and its pH was 5·86. The purple colour was discharged on acidification and became yellow. The solution rapidly reduced permanganate and gave a brown colour with ferric chloride. 50 ml. liberated iodine, from acidified KI solution, equivalent to 4·87 ml. $N/100$ $Na_2S_2O_3$.

Isolation and purification of spinulosin

The filtered metabolism solution from 90 flasks was acidified by the addition of conc. HCl (20 ml./l.) and was then extracted twice, in 2 l. lots, with an equal volume of ether, the ether used for the second extraction of one lot of metabolism solution being used again for the first extraction of another lot. The ethereal extracts were washed with a little water, dried over anhydrous $MgSO_4$ and evaporated to about 1 l. This ethereal solution was now extracted 4 times with a total volume of 200 ml. of a buffer solution (pH 7·0) made by mixing 50 ml. M KH_2PO_4 with 29·6 ml. N NaOH and diluting with water to 100 ml. The extracted ethereal solution was redried and evaporated to dryness, giving 1·7 g. of a gummy residue. No evidence of the presence in this fraction of dihydrospinulosin could be obtained, and nothing crystalline could be isolated except 0·15 g. of an unidentified yellow solid, M.P. 185–190°. The combined buffer solution extracts, which were deep purple in colour, were acidified with conc. HCl and extracted with ether: the ethereal extract was dried and evaporated, giving 2·3 g. of a crystalline, slightly sticky, purple-black residue of crude spinulosin. This was purified by crystallization from toluene, followed by sublimation in a high vacuum and finally by recrystallization from toluene. The final product (0·93 g.) was obtained as lustrous purple-black plates and was identified as spinulosin by its M.P. 200°, alone or in admixture with an authentic specimen, by its colour reactions (a deep pure blue with cold conc. H_2SO_4, a bluish-purple with $2N$ NaOH, and a very dark rich brown with $FeCl_3$ in alcohol), and by analysis. (Found (Weiler): C, 52·32, 52·39; H, 4·34, 4·40; $O.CH_3$, 16·9, 16·85 %. Calc. for $C_8H_8O_5$: C, 52·17; H, 4·38; 1 $O.CH_3$, 16·9 %.)

SUMMARY

Spinulosin (3:6-dihydroxy-4-methoxy-2:5-toluquinone), previously reported as a metabolic product of *Penicillium spinulosum* Thom, has now been isolated from cultures of a strain of *Aspergillus fumigatus* Fresenius. This finding contrasts with the fact that a different strain of *A. fumigatus* gives fumigatin (3-hydroxy-4-methoxy-2:5-toluquinone).

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CCXCIII. THE DISSOCIATING POWER OF SALTS OF FATTY ACIDS

PRELIMINARY PAPER

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THIS work had its origin in the observation that the dissociative change in colour induced in lobster shell by alcohol was partly reversible. This was made shortly before the appearance of the preliminary paper of Stern & Salomon [1937], who have drawn attention to the reversible colour change occurring upon mild heating of the chromoprotein, ovoverdin, from lobster eggs, and have explored part of this interesting field [1938]. Denaturation of ovoverdin, as ordinarily seen, involves the two processes (*a*) of dissociation of the astacin ester from the associated protein, giving the colour change green to red, and (*b*) of denaturation (with coagulation) of the protein itself. We deal here only with the dissociation, making the assumption that the colour change represents a change in the state of combination of the carotenoid with the protein. We found that this dissociation can be quickly induced at neutral reaction by traces of the Na salts of oleic and of other fatty acids, and further that these acids, when added to a respiring brain *brei*, inhibit respiration. It is suggested here that the two sets of observations are related, and that this inhibition is caused by an adsorptive dissociation of the prosthetic groups of certain enzymes from the apoenzymes.

Few investigations have been found of the general problem of the action of soaps upon enzymes. Velluz [1927] stated that fatty acids inhibit the action of pepsin, the effect increasing from C_6 to C_{18}; unsaturated fatty acids were more potent than the saturated ones, though with urease the latter were more active. Related to the question also seems to be the finding of Quastel [1932] that unsaturated glycerides increase the toxicity of the triphenylmethane series of dyes towards urease, though this action was rather specific.[1]

There is an evident relation also to the film penetration observations of Schulman & Rideal [1937] and Schulman [1937], though the nature of this relation is not yet defined.

EXPERIMENTAL

Ovoverdin. This was extracted from lobster eggs [Stern & Salomon, 1937] by grinding with sand and water and treatment of the fluid portion with an equal volume of saturated ammonium sulphate. After filtration, the ovoverdin was precipitated from the filtrate by more complete saturation with ammonium

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[1] Upon completion of this work, there appeared a note in which Cook & Kreke [1938] state that fatty acids in a concentration of 0·5 mg./ml. stimulate the respiration of yeast, and at 1 mg./ml. stimulate skin respiration slightly. Higher concentrations were inhibitory. Such amounts cause massive depression in the pyruvate system here studied; a special further test showed that neither 0·1 mg. nor 0·05 mg. Na decoate had any stimulating effect with brain though these lie immediately below the concentration which has an inhibitory action, viz. 0·2 mg./3 ml.

sulphate, the precipitate collected upon a fluted filter paper, under cool conditions, and dissolved in 1 % NaCl. Traces of ammonium sulphate were removed by dialysis against 1 % NaCl. It was found to be important to keep the chromoprotein moist at all stages with dilute salt solution; drying or approximation to salt-free solutions rapidly causes the dissociative change from green to red. It is interesting in view of what follows that in its relation to salts and drying the chromoprotein follows closely in stability the pyruvate oxidase system in brain tissue, though there is a difference in sensitivity to freezing. This does not appear to affect ovoverdin, whereas it quickly destroys pyruvate oxidase.

Na salts of fatty acids. With the exception of the Na palmitate and oleate, which were of doubtful purity, the remaining soaps were obtained from commercial sources, but were subjected to several recrystallizations. The C_{10}, C_{12} and C_{14} acids were prepared from acids previously purified to the correct M.P. Some difficulty is experienced in obtaining solutions of the less soluble salts at the reaction required for test, approximately pH 7·4. It will be realized from the study of the pH relations at interfaces of the fatty acids [Peters, 1931; Wakelin, 1938] that at this reaction dodecoic acid tends to be present mainly as the acid and to leave an aqueous phase; decoate on the other hand shows a more marked water-solubility. Hence the use of a soap at this reaction must in reality mean the use of a mixture of salt and acid, and the really important point is to add it in a sufficiently soluble form to ensure that the mixture reaches the enzyme studied. In practice with dodecoate, tetradecoate and palmitate after solution in the appropriate amount of water, the whole was warmed, traces of phenol red added, and then HCl to give the desired pH. Where solution was possible only at 38°, the soap solution was kept in the warm bath until addition, together with a control containing water only. The soap and the water control were then added in similar volumes as a final addition to the Barcroft bottles. The highest concentrations used were Na octoate 0·1 M, Na decoate 0·045 M, Na dodecoate 0·047 M and Na tetradecoate 0·00206 M.

In other respects the respiration experiments with pigeon's brain tissue followed the usual technique of the laboratory; it will be recalled that the Ringer-phosphate medium (pH 7·3) does not contain Ca. The experimental volumes were 3·0 ml. in the Barcroft bottles; $1·504 \times 10^{-3}$ M Na decoate = 1 mg. in 3·0 ml. In most experiments the figures quoted are the mean of duplicate estimations. The vitamin added was enough to produce maximum effects, 1·0–2·0 μg.; Na pyruvate, 6 mg. was added to each bottle.

Effect of Na salts upon ovoverdin

The minimum amount of Na salt necessary to cause dissociation was found by adding it in solution to 1·0 ml. of a suitable dilution of ovoverdin in 1 % NaCl or in phosphate buffer (pH 6·7), until the green colour had disappeared. Table I shows the results with buffer present: in absence of the latter, the results were 20 % higher but in the same sense.

Table I. *Amounts of Na salts of fatty acids required to cause dissociation of dilute ovoverdin solutions. Temp. 16–20°*

	$\times 10^{-3}$ M
Na octoate	>12·8
Na decoate	4·17
Na dodecoate	1·105
Na tetradecoate	1·042
Na palmitate	<1·2
Na oleate	1·09

The optimum concentration is reached with C_{12}; the difference between this and C_{14} is not significant. The figure for palmitate is unreliable owing to the low solubility. Since the change from water-solubility to lipoid-solubility takes place with a length of C chain of about 10, it seems to be clear that the effect is correlated with adsorption at the surface of an internal phase, and is explained if the acid displaces the astacin ester from its combination.

Reversibility. We may ask whether the acid interferes by combining irreversibly, or whether the effect is a loose displacement. It was not found possible to extract the fatty acid by any means which would leave the protein undenatured; but a simple addition of $CaCl_2$ to 0.02–0.04 M readily causes precipitation of such fatty acids under these conditions, and in the absence of phosphate will reverse the colour change to green. This is quite consistent with the idea that we are dealing with a reversible adsorptive phenomenon.

Effects upon some oxidation enzyme systems

The above observations are of interest in themselves, but they gain in importance by the finding that a similar action takes place upon a brain *brei*. The question has not been thoroughly explored; only with pyruvate and succinate as substrates has it been investigated, and with the latter not in detail. The interesting point is that with pyruvate there is inhibition increasing from the C_8 acid to the C_{12}, as with the dissociation of the lobster pigment. Fig. 1 illustrates the action of sodium decoate. In Table II the results for pyruvate are collected under the headings of the acid to which they refer. Where the actions of more than one acid were compared upon the same brain this may be seen from the no. of the exp. (column 6, Table II). In general the results for different exps. seemed to be sufficiently similar to make this the best method of examination. In the summary (Table II), the essential point is made that the inhibitory action increases from C_8 to C_{12}; after this the results for tetradecoate are of the same order, but are slightly complicated by solubility. Palmitate seems to be less active but the result is again rather uncertain, and the figure for concentration can only be regarded as a maximum one. With decoate and dodecoate amounts of 0.2–0.3 mg. produce definite effects. It is suggested in explanation that the acids displace an essential component of the oxidase system, as with the model ovoverdin. The experiments have been done throughout upon the avitaminous brain in order to obtain possible extra information; they are not complicated by the possibility that time was not allowed for combination of vitamin, because at least 5 min. were allowed after addition of vitamin and before adding the fatty acid. In many cases the inhibition for vitamin is greater than that for pyruvate alone; but this is probably not significant, because the figure for respiration without added vitamin has a relatively larger contribution from the residual respiration, which appears to be less inhibited.

In support of the idea that some coenzyme may be displaced from combination by the acid, we have found that with 4.51×10^{-8} M decoate the CO_2 evolution from pyruvate by washed yeast (acetone-treated) with added cocarboxylase is reduced by 35% both alone and in the presence of $10\,\mu g$. vitamin B_1; this experiment was carried out as described by Ochoa & Peters [1938].

The oxidase system for succinate in brain is more stable than that for pyruvate; the former survives freezing and extraction with distilled water. Similarly it is not much affected by fatty acid; a complete experiment upon this is given in exp. 12 with dodecoate; similar results have been obtained with decoate.
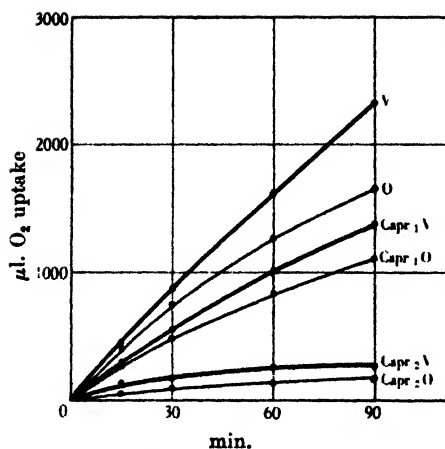
Fig. 1. The effect of small amounts of Na decoate upon the O_2 uptake of avitaminous pigeon brain in Ringer-phosphate pyruvate medium. Upper curves: V = with and O = without vitamin. Middle curve (capr.$_1$), addition of 0·2 mg. decoate. Lower curves (capr.$_2$), 0·7 mg. decoate.

Table II. *Inhibiting effect of Na salts of fatty acids upon rate of the O_2 uptake of pigeon's brain tissue (in presence and absence of vitamin B_1) with pyruvate, expressed as a decrease in $\mu l./g./hr.$ and as a % decrease.*

	Conc. $M \times 10^{-3}$	No vitamin		Vitamin		Exp.
		μl.	%	μl.	%	
Octoate	16·0	− 905	64·5	− 1575	71·6	5
	4·8	− 256	18·0	− 851	38·8	5
	1·9	+ 39	Nil	− 357	18·4	6
	1·6	+ 43	Nil	− 265	12·0	5
Decoate	5·27	− 848	88·7	− 1586	94·1	1
		− 832	92·6	− 1375	94·1	2
	4·51	− 646	82·5	− 1314	89·5	3
	1·5	− 520	40·6	− 1182	55·4	6
		− 485	48·5	− 1007	59·0	7
	1·05	− 282	14·6	− 621	42·5	2
	0·31	− 3	Nil	− 210	14·4	2
		− 32	4·0	− 207	14·0	3
Dodecoate	4·94	− 753	83·9	− 1262	82·9	2
	1·65	− 774	77·4	− 1341	78·6	7
	0·99	− 325	38·5	− 674	43·6	8
	0·424	− 100	11·9	− 312	20·2	8
Tetradecoate	0·67	− 211	31·2	− 516	33·6	10
	0·13	− 57	8·5	− 315	20·4	10
	0·33	− 28	3·5	− 175	13·3	11
	0·065	− 115	15·0	− 160	12·1	11
Palmitate	0·6	− 79	10·6	− 226	15·8	9
	0·12	+ 18	Nil	− 102	7·2	9
Oleate	1·97	− 578	60·5	− 1217	72·2	1
	0·77	− 523	54·7	− 917	54·4	1

The periods of respiration after start of the exp. upon which the above figures are based are as follows: 30–60 min., exps. 5, 7; 30–90 min., exps. 1, 2, 3, 6, 9; 30–120 min., exps. 8, 10, 11.

Summary

Acid	Conc. $M \times 10^{-3}$	Decrease %	
		O	V
C_{12}	1·65	77·4	78·6
C_{10}	1·5	48·5	59·0
C_8	1·6	Nil	12·0

Exp. 12. Avitaminous brain used so as to eliminate pyruvate oxidation to some extent in the residual oxidation. The figures quoted are for the period of respiration 15–90 min. NaD = Na dodecoate.

Substances present	Rate of resp. μl./g./hr.	Difference
No addition	968	
Succinate	2566	1598
0.705×10^{-3} M NaD	470	
Same + Succ.	1922	1452
1.41×10^{-3} M NaD	323	
Same + Succ.	1662	1339

Na succinate 0·082 M. Note that 1.65×10^{-3} M reduces respiration with pyruvate 77 % and 0·99, 38 %. The decrease in the residual oxidation was here 52 and 66 % respectively.

As would be expected from this, the succinic dehydrogenase from liver (pigeon) showed no reduction in activity upon adding Na dodecoate ($3.4 \times 10^{-3} M$). On the other hand exp. 13 shows that the system which causes dehydrogenation of pyruvate in brain is much affected.

Exp. 13. *Inhibition of dehydrogenase action by Na dodecoate.* The tissue from two normal pigeon brains was thoroughly mashed and extracted 6 times with 7 ml. 1 % KCl and 4 times with 7 ml. Ringer phosphate, *p*H 7·4. Care was taken to grind the whole thoroughly with a glass rod and to keep ice cold throughout but not frozen. Thunberg tubes were set up containing 50 mg. tissue, 0·2 ml. methylene blue (1:5000), 3 mg. Na pyruvate and 1·0 ml. Ringer-phosphate. The Na pyruvate was placed in the stopper and added after evacuation just before placing in the bath at 38°. One tube contained in addition 1 mg. Na dodecoate. Figures are quoted for one out of four similar experiments.

	Time for decoloration
Control	30 min.
Na pyruvate	9 min.
Na pyruvate with Na dodecoate	> 150 min.

The results for the pyruvate dehydrogenase of pigeon's brain confirm those of Lipmann [1937]. It will be seen that the dodecoate has inhibited not only the pyruvate effect but also the residual reduction.

It might be thought that some of the phenomena described above were merely the result of the well-known cytolysing action of soaps, and consequent destruction of enzyme action intimately bound up with cell structure. This clearly could not apply to the ovoverdin, nor to the acetone-treated yeast; in the case of the pyruvate oxidase system, Dixon & Meyer [1936] have shown that many brain cells are destroyed in forming a *brei*; in confirmation and extension of this Dr Carleton has found that, in our brain *brei*, the cytoplasmic outlines of the cells are destroyed (unpublished). Hence the evidence is that here too the effect is on the enzyme system itself rather than the cell.

DISCUSSION

There seems to be no doubt as to the practical importance of these observations. Where there is a possibility that fatty acids may pre-exist or be formed by lipase action or otherwise during the course of experimental procedure, it seems certain that they will influence adversely experimental results with isolated systems. In particular it is likely that they constitute some of the difference between the tissue *brei* and the slice. The insolubility at *p*H 5·5–6·0 of the inhibitor of brain glycolysis described by Geiger [1938] suggests an effect of this type. Again, this may be part of the reason why Quastel & Wheatley [1933],

and Jowett & Quastel [1935] found oxidation of fatty acids by slices of liver, but none with *brei*. If there is substance in the idea that the behaviour of ovoverdin serves as a model for the more loosely associated groups of enzymes, a new experimental tool is indicated. It has been pointed out previously [Peters, 1931] that the fact that fatty acids of the water-insoluble type ionize over the physiological range of pH is of biological significance. Here we find that part of the colloidal phase of a chromoprotein in solution is affected by the same type of acid. This shows that such interfaces exist in practice in such solutions. Further it is clear that the cell possesses yet another simple method of controlling its activities.

SUMMARY

Small amounts of the Na salts of saturated fatty acids (C_{10}, C_{12}, C_{14}) cause dissociation of the astacin ester from the protein compound in ovoverdin; this change can be reversed by Ca salts.

Similarly these soaps exert a markedly inhibitory action upon the pyruvate oxidase system; the effect upon the succinoxidase system is much less.

It is suggested that these phenomena are related.

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CCXCIV. S-CYSTEINOSUCCINIC ACID

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WE have reported previously [Morgan & Friedmann, 1938, 1] that thiol compounds react with maleic acid giving stable condensation products of the general formula $HOOC.CH_2.CH(COOH).SR$. Of the three substances investigated, thiolacetic acid, *l*-cysteine and glutathione, only thiolacetic acid gave an easily crystallizable reaction product. We have now succeeded in crystallizing the product obtained by the interaction of *l*-cysteine with maleic acid.

The new substance which we call S-*cysteinosuccinic acid* separates from methanol in hedgehog crystals, M.P. 134–135° after softening at 102° and from glacial acetic acid in needles, softening at 125° and melting at 134–135°. The analytical figures of both products were in agreement with the formula $C_7H_{11}O_6NS$, but the optical rotations were different. The substance from methanol was laevo-rotatory in aqueous solution whilst that from glacial acetic acid was optically inactive. It was found in fact that hot glacial acetic acid induced partial or complete racemization; thus the optical rotation of the laevorotatory substance from methanol ($[\alpha]_D = -29\cdot8°$), after one recrystallization from glacial acetic acid decreases considerably ($[\alpha]_D = -5\cdot1°$) and becomes zero after a second recrystallization from the same solvent. Simultaneously the form of the crystals changes from rosettes to grid needles.

The addition of *l*-cysteine to maleic acid involves the formation of a second asymmetric centre (**):

$$HOOC.CH_2.\overset{**}{C}H.(COOH).S.CH_2.\overset{*}{C}H(NH_2).COOH,$$
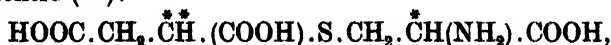
so that two optically active condensation products can result. So far we have no experimental evidence for the actual formation of a second optically active compound. The laevorotatory substance described in this paper is certainly the main, if not the only, reaction product. It can be isolated in a yield of 86·75 % from the amorphous product resulting (93·5 % yield) from the interaction of *l*-cysteine with maleic acid [Morgan & Friedmann, 1938, 1].

The directing influence of the first asymmetric carbon atom (*) on the configuration of the newly formed second asymmetric carbon atom (**) is in agreement with well known stereochemical observations. The actual configuration of this second asymmetric carbon atom (**) however must at present remain open.

The maleic acid reaction has been applied by us [Morgan & Friedmann, 1938, 2] to the study of enzyme reactions induced by SH-compounds. In the discussion of these results we pointed out that the conclusions based on the inhibition of an enzyme reaction by maleic acid must be checked by a direct method. We suggested Hopkins's SS, SH reaction [Hopkins, 1921] as suitable for this purpose [Hopkins & Morgan, 1938]. Recently v. Euler & Hellström [1938] have raised objections against the validity of conclusions based on Hopkins's reaction. We tried to supply the missing direct chemical evidence for an assumed interaction of maleic acid with SH-groups of enzymes or coenzymes. As SH occurs naturally, so far as we know at present, only as free

or combined cysteine, *S*-cysteinosuccinic acid should be formed by hydrolysis of enzymes or coenzymes after their reaction with maleic acid. This view has been shown to be correct for *S*-glutathionosuccinic acid. Hydrolysis of this compound gave *S*-cysteinosuccinic acid. The product was partially racemized.

EXPERIMENTAL

Crystallization of S-cysteinosuccinic acid from methanol

(1) 0·4 g. amorphous condensation product of *l*-cysteine and maleic acid [Morgan & Friedmann, 1938, 1] was dissolved in hot methanol. On cooling the solution slowly deposited white crystals. After removal of the supernatant fluid the crystals are washed with methanol and dried *in vacuo* at 45° over P_2O_5. (Found: C, 35·1; H, 4·6; N, 5·6; S, 13·1 %. $C_7H_{11}O_6NS$ requires C, 35·5; H, 4·6; N, 5·9; S, 13·5%.) $[\alpha]_D^{21°} -29.75°$, $-29.84°$ ($c = 2.236$ in water).

(2) 0·4 g. amorphous *S*-cysteinosuccinic acid was dissolved in approximately 30 ml. of hot methanol. Next day the solution was slowly taken down to 4 ml. under slightly reduced pressure. 0·347 g. of white hedgehog crystals separated which were washed with methanol. Yield 86·75%. $[\alpha]_D^{23°} -27.58°$ ($c = 2.357$ in water).

S-Cysteinosuccinic acid commences to shrink and soften at 102° and melts at 134–135° (decomp.). It is hygroscopic and the M.P. had to be taken after drying the substance in the melting tubes for several days *in vacuo* over P_2O_5. The substance is easily soluble in cold water, slightly soluble in cold, better in hot methanol. In hot glacial acetic acid it dissolves slowly and sparingly, apparently with decomposition which turns the solution yellow. It is not soluble in the other usual organic solvents.

Effect of glacial acetic acid on crystalline, laevorotatory S-cysteinosuccinic acid

(1) 0·1 g. crystalline *S*-cysteinosuccinic acid ($[\alpha]_D = -29.8°$) was dissolved in boiling glacial acetic acid. On cooling 0·0745 g. of crystals deposited quickly as a mixture of forms mostly needles with some rosettes $[\alpha]_D^{21°} -5.12°$ ($c = 2.246$ in water).

Another sample prepared in the same way was analysed after drying *in vacuo* over P_2O_5 and KOH at 40°. (Found: C, 35·9; H, 4·8; N, 5·7; S, 12·8%. $C_7H_{11}O_6NS$ requires C, 35·5; H, 4·6; N, 5·9; S 13·5%.)

(2) 0·1 g. substance ($[\alpha]_D = -29.8°$) was dissolved in boiling glacial acetic acid and allowed to crystallize overnight. The crystals were redissolved in the mother liquor by boiling. On cooling and standing overnight 0·0430 g. needles of uniform appearance were obtained. The mother liquor was deep yellow and gave on concentration a gummy residue. M.P. of the needles was 134–135° (decomp.) after softening at 125°. $[\alpha]_D$ in water was zero.

The substance used for polarimetry was recovered and analysed after drying as above. (Found: C, 35·5; H, 5·0%. $C_7H_{11}O_6NS$ requires C, 35·5; H, 4·6%.)

S-Cysteinosuccinic acid obtained by hydrolysis of S-glutathionosuccinic acid

S-Glutathionosuccinic acid [Morgan & Friedmann, 1938, 1] was hydrolysed by Hopkins's [1929] method used for the hydrolysis of glutathione.

0·5 g. substance was boiled with 25 ml. of 25 % H_2SO_4 for 16 hr. The solution, diluted to 800 ml., was precipitated with $HgSO_4$ avoiding excess. The Hg precipitate separated slowly. After 2 days it was collected and decomposed with H_2S. H_2SO_4 was removed from the filtrate quantitatively with $Ba(OH)_2$, the solution transferred to a crystallizing dish and evaporated *in vacuo* over

H_2SO_4. Yield 0·21 g. Recrystallized from hot methanol, 0·090 g. hedgehog crystals were obtained. M.P. 134–135° after softening at 102°. For analysis the substance was dried *in vacuo* over P_2O_5 at 45°. (Found: C, 35·2; H, 4·8; N, 5·6 %. $C_7H_{11}O_6NS$ requires C, 35·5; H, 4·6; N, 5·9 %.) $[\alpha]_D^{20°} -6·43°$ ($c = 1·786$ in water).

The properties of the isolated substance were identical with those of *S*-cysteinosuccinic acid.

SUMMARY

S-Cysteinosuccinic acid crystallizes from methanol in hedgehog crystals, softening at 102° and melting at 134–135° (decomp.).

On treatment with hot glacial acetic acid racemization occurs, apparently accompanied by partial decomposition.

S-Cysteinosuccinic acid is formed by hydrolysis of *S*-glutathionosuccinic acid.

The significance of *S*-cysteinosuccinic acid for the interpretation of the maleic acid reaction in enzyme work is pointed out.

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CCXCV. INTERRELATIONS OF THE VITAMINS

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In recent years the possibility of an antagonism between vitamins A and C has often been mentioned. This antagonism, if it exists, might be revealed by the partial inhibition of the effects of vitamin C in scurvy by the simultaneous administration of vitamin A.

During the last few years we have had the opportunity of making a close study of vitamins A, B complex, C and D, and being somewhat sceptical about the existence of the above-mentioned antagonism we decided to make a thorough investigation of the subject. We wish to make it clear that when we speak of antagonism we have in mind harmful antagonism.

It is generally agreed that a balance should exist between the various vitamins. A state of equilibrium can be preserved in spite of considerable variations in the amounts of the vitamins supplied, but if one particular vitamin is present in too great an excess, the symptoms of hypervitaminosis will be the consequence. In such cases it may be possible to restore the health of the organism by supplying an adequate quantity of one or more of the other vitamins. The equilibrium between the vitamins is now re-established by the increased supply of the relatively deficient vitamins, an example of beneficial antagonism.

The existence of beneficial antagonism between the fat-soluble vitamins A and D on the one hand, and the water-soluble vitamins B and C on the other, appears to be unquestioned. Publications by workers such as Hopkins [1923], Harris & Moore [1928], von Euler [1933], Höjer [1926] and Light *et al.* [1929], reveal clearly its existence. We have therefore considered it superfluous to investigate this problem and have limited ourselves to a study of the existence of the possible harmful antagonistic action of vitamins A and D on vitamin C.

In studying the available literature in order to establish the grounds for the belief that there is a harmful antagonism between vitamins A and C, we have succeeded in finding only one publication which in our opinion seems to be reliable and which might be construed as revealing such an antagonism. This publication is by Mouriquand & Michel [1922], but even here there is no question of an actual antagonism between vitamins A and C, because these workers rightly limit themselves to the demonstration that cod liver oil under given conditions may have a destructive action on the antiscorbutic effect of vitamin C. We have therefore come to the conclusion that there is no evidence for such antagonism in the case of either man or the lower animals. On the other hand, there is evidence in favour of the existence of a synergistic action between these vitamins. Thus we might mention innumerable cases in which physicians prescribe cod liver oil and orange juice for infants and also the unquestionably good results obtained with various commercial preparations containing vitamins A and C.

Our experiments were carried out with a liquid "Preparation X", composed chiefly of a concentrate of vitamins A and D, malt extract, orange juice, sugar and water. 1 ml. of this product (= 1·32 g.) contained 800 I.U. vitamin A (i.e. 600 I.U. per g.), 120 I.U. vitamin D, between 5 and 5·5 I.U. vitamin C and also a little of the vitamin B complex. The ratio of vitamin A to vitamin C was therefore 800 to 5·3 or 150 to 1 in terms of International Units. Preparation X contained about 1 % of the above-mentioned vitamin concentrate.

Three series of preliminary experiments were made; in each series there were three groups of guinea-pigs, with four animals in each group.

The main object was to compare "Preparation X", which contained vitamins A and D as well as vitamin C, with "Preparation Y" which differed from Preparation X only in the fact that it contained no vitamin A or D. It was desired to ascertain whether vitamins A and D in Preparation X would inhibit the action of the vitamin C which it contained.

The daily diet in the three groups is shown in Table I.

Table I

Diet	Added vitamins	Ratio vitamin A : vitamin C
(1) Basal diet + 4 g. Prep. X	2400 I.U. vitamin A + 16 I.U. vitamin C	150 : 1
(2) Basal diet + 4 g. Prep. Y	23 I.U. vitamin A + 16 I.U. vitamin C	1·43 : 1
(3) Basal diet alone	No vitamin C	—

The basal diet was that of Tillmans and consisted of oats, milk, hay and a little cod liver oil, equivalent to a dose of 23 I.U. vitamin A and 3 I.U. vitamin D per diem. Preparations X and Y were given mixed in milk.

The animals in Groups 1 and 2 were fed on the basal diet alone until increase in weight ceased or loss of weight took place and were then given the preparations. Weights were recorded twice a week over a period of 50 days except in the case of Group 3 when the death of the animals occurred before the close of the testing period.

Following the preliminary experiments in which only a small number of animals was used in each group, work was carried out with a series composed of four groups with 10 animals in each group.

In order to compare the action of cod liver oil with that of Preparation X an extra group was included in this series. The animals in this extra group received the same quantities of vitamins A, D and C as those of the group receiving Preparation X, but vitamins A and D were given in the form of cod liver oil. Vitamin C was administered in the same way as in Group 2, namely in Preparation Y. Another reason for introducing this extra group fed on cod liver oil was to verify the experiments of Mouriquand & Michel [1922] in which they had used a basal diet with added orange juice and cod liver oil.

In order to have the same quantity of vitamin A (2400 I.U. vitamin A) in proportion to vitamin C as in the group fed on Preparation X, and so that the quantities of cod liver oil and of Preparation X should be of the same magnitude, we used 4 g. cod liver oil containing 600 I.U. vitamin A per g. The basal diet was the same as before. The cod liver oil was converted into an emulsion and mixed with the milk given to the animals.

The action of the vitamin C administered was assessed on the one hand by the increases in weight and the general condition and appearance of the animals, and on the other hand by post-mortem examinations of the animals which died or were killed and by X-ray photographs of the thorax.

We have prepared graphs showing the mean variations in weight for the groups of animals in each experiment, that is to say for the Preparation X group, Preparation Y group, and the negative control group in each of the three experiments of the preliminary series (see Figs. 1, 2 and 3) and for the Preparation X group, Preparation Y group, Y with cod liver oil group and the negative control group in the larger-scale experiment (see Fig. 4).

One animal fed on Preparation Y died from pneumonia. Three of the animals receiving cod liver oil died, probably from scurvy (post-mortem examination revealed scorbutic signs in each case). The negative control animals all died in less than 50 days with typical symptoms of scurvy.
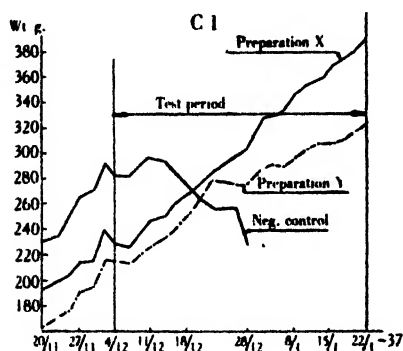
Fig. 1.
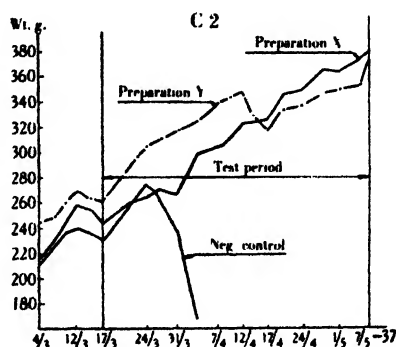
Fig. 2.
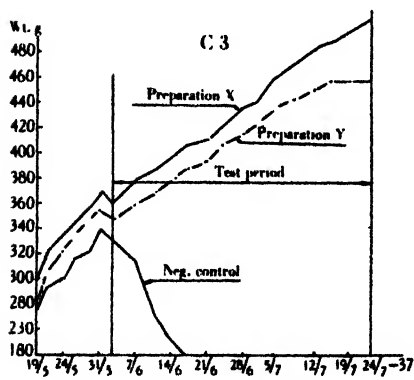
Fig. 3.
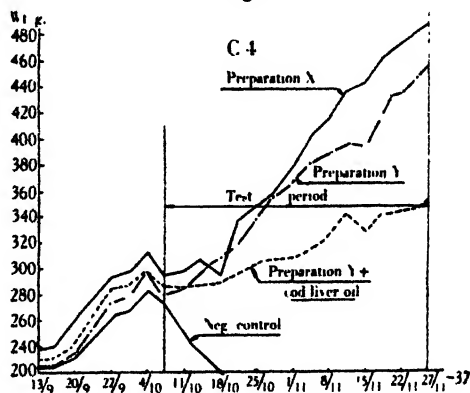
Fig. 4.

As can be seen from the curves, the mean increase in weight was in every series a little greater for animals receiving Preparation X (including vitamins A and D) than for those receiving Preparation Y (without added vitamins A and D). If, then, increase in weight is taken as a criterion of the effect of vitamins A and D on the action of vitamin C, the results of every experiment seem clearly to indicate that there is no antagonism between vitamins A and D and vitamin C. It will be noted that the weight increase of the animals receiving cod liver oil (Fig. 4), was less than that of those animals receiving Preparation X or Y.

In spite of the very large amount of vitamin A administered, large both from an absolute point of view and in proportion to the amount of vitamin C,

hypervitaminosis-A did not occur and the vitamin A appears to have reinforced the action of vitamin C.

The general appearance of the animals of the X groups was excellent, that of the Y groups very good, that of the cod liver oil group bad and the animals of the negative control groups were of course miserable in appearance.

Results of the autopsies

After the animals had died or had been killed at the end of the testing period, observations were made regarding the existence of haemorrhages and fragility of the bones and the condition of the thorax was examined. The results of these examinations are given below (Table II).

Table II. *Results of autopsies*

Series no.	Group no.	Preparation	Haemorrhages	Bone fragility	Scorbutic rosary
I	1	X	None	Normal	None
	2	Y	Almost none	Normal	Traces
	3	Negative control	Severe	Very fragile	Pronounced
II			Exactly as for Series I		
III			Exactly as for Series I		
IV	1	X	None	Normal	None
	2	Y	Almost none	Normal	Traces
	3	Y + cod liver oil	Rather pronounced	Rather fragile	Rather pronounced
	4	Negative control	Severe	Very fragile	Pronounced

All the animals in Group 1 were plump and healthy and the carcasses were light in colour.

The animals belonging to Group 2 (Preparation Y) were likewise in good condition, but they showed a few small haemorrhages, the colour of their carcasses was not so light as in Group 1, neither were they quite so plump. The bones were strong. X-ray examination of the thorax did not give quite so favourable a picture as in the case of Group 1. It could not be said that animals belonging to Group 2 exhibited distinct scorbutic symptoms and the difference between those receiving Preparation X and those receiving Preparation Y was very slight on the average. Any difference that existed was in favour of the " X " animals in Group 1.

All the negative control animals (Group 3 in the preliminary series I, II and III and Group 4 in the "Large Series" IV) showed typical symptoms of scurvy, severe haemorrhages, fragile bones (sometimes even breaking before death) and a pronounced scorbutic rosary.

Animals belonging to Group 3 in the "Large series" (cod liver oil) also showed symptoms resembling those of scurvy.

Mouriquand & Michel [1922] have shown that cod liver oil, under the conditions obtained in trials with guinea-pigs, can inhibit the action of ascorbic acid. However, we agree fully with the reservations made by them that one cannot conclude from these experiments on guinea-pigs that cod liver oil given in ordinary doses inhibits the antiscorbutic action of vitamin C in man. In this connexion it is interesting to note that Wieland [1936] denies the existence of any antagonism between vitamins A and C in human beings. It must be remembered that the doses of cod liver oil administered to the guinea-pigs in our experiments were extremely large. Taking into consideration the relative weights of the animals and man, the corresponding dose for a man would amount to nearly 1 kg. cod liver oil a day.

Stability of vitamin C in presence of vitamins A and D

It has been claimed that vitamin C may be destroyed by vitamin A *in vitro*. We have examined the chemical relations between vitamins A and D on the one hand and vitamin C on the other, using emulsions corresponding in their make-up to Preparations X and Y. The results of such investigations will not be given in detail on this occasion but they showed that vitamin C was not appreciably reduced in amount in either preparation 3 months after the date of their manufacture. After an interval of 6 months the diminution was 10–15 %. These tests were made with a commercial preparation and no special precautions had been taken to prevent the destruction of vitamins. The analyses were made by Tillmann's method. Thus it was impossible to prove that vitamin C had been affected by the presence of vitamins A and D in the preparation.

SUMMARY

1. Using guinea-pigs as experimental animals it was found that vitamins A and D had no antagonistic action on vitamin C, even if the doses of the fat-soluble vitamins were very large in relation to the dose of vitamin C.

2. It seems possible that vitamins A and D can reinforce the action of vitamin C, even if the excess of vitamins A and D over vitamin C is very considerable.

3. Under the conditions of our *in vitro* experiments vitamins A and D had no destructive chemical action on vitamin C.

4. Very large doses of cod liver oil inhibited the antiscorbutic action of vitamin C in guinea-pigs.

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